THE EFFECT OF CERTAIN SURGICAL ANTISEPTICS AND THERAPEUTIC AGENTS ON PHAGOCYTOSIS. I. CARBOLIC ACID, MERCURIC CHLORIDE, BORIC ACID, QUININE HYDROCHLORIDE.[1]

By WILFRED H. MANWARING and HAROLD O. RUH.

(*From the Pathological Laboratory of Indiana University.*)

The present wide interest in opsonotherapy and belief in the importance of phagocytosis in immunity, make it desirable to determine the effect of the commoner medicinal agents on phagocytic power. The effect of the three most important surgical antiseptics, namely, mercuric chloride, carbolic acid and boric acid, and of the most widely used internal remedy in acute infections, quinine hydrochloride, have thus far been determined, and are here presented.

Material and Technique.—Except in the one experiment hereinafter specially mentioned, the material used in this study was defibrinated human blood and streptococci suspended in physiological saline (0.85 per cent. sodium chloride). The streptococci were in all cases obtained from twenty-four to forty-eight hour cultures on blood serum (goat), sterilized at 100° C.

To test the effect of a chemical substance on phagocytosis, increasing amounts of a solution of that substance (whenever possible equimolecular with 0.85 per cent. sodium chloride) were added to a number of test-tubes, and the volumes in the tubes made constant (1 c.c.) with physiological saline. There was then added to each tube a constant amount (1 c.c.) of freshly drawn, defibrinated blood and, at stated intervals, a constant volume (1 c.c.) of streptococcus suspension.

Each tube was immediately immersed in a thermostatic water bath at 37.5° C., and fifteen, thirty and sixty minutes later, smears

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were made from it. These smears were stained by Wright's method and the number of bacteria in sixty polymorphonuclear leucocytes in each smear counted.

Experimental Error.—In beginning quantitative work in any field of biological chemistry, it is necessary, first of all, to determine the accuracy of the proposed experimental method—the limits of the experimental error. Without such determination, deductions from experimental data are comparatively valueless. An experiment was therefore undertaken to determine the range of error in work by the above technique.

To do this, ten duplicate tubes were prepared, each containing a mixture of equal parts (1 c.c.) of physiological saline, defibrinated human blood and streptococcus suspension. Smears were made from these tubes and counted, exactly as in the proposed investigation.[2] The data from this experiment are given in Table I.

TABLE I.

Duplicate Bacterial Counts.

		1	2	3	4	5	6	7	8	9	10	Average.
15 Minute Counts	Wright's Index	5.63	5.58	6.18	5.23	5.87	5.83	5.72	5.27	5.53	5.21	5.61
	Error	+ .02	− .03	+ .57	− .38	+ .26	+ .22	+ .11	− .34	− .08	− .40	± .24
	Percentage Error	+ 0.4	−0.5	+10.0	−6.8	+4.6	+3.9	+2.0	−6.1	−1.4	−7.1	±4.3
30 Minute Counts	Wright's Index	[9.63]	8.40	7.65	7.48	7.38	7.35	7.88	8.15	7.83	7.50	7.73
	Error	[+1.90]	+ .67	− .08	− .25	− .35	− .38	+ .15	+ .42	+ .10	− .23	± .29
	Percentage Error	[+24.6]	+8.7	−1.0	−3.2	−4.5	−4.9	+1.9	+5.4	+1.3	−3.0	±3.8
60 Minute Counts	Wright's Index	12.60	11.80	12.15	12.05	11.93	11.35	11.48	10.87	11.03	11.08	11.64
	Error	+ .96	+ .24	+ .51	+ .41	+ .29	− .29	− .16	− .77	− .61	− .56	± .48
	Percentage Error	+ 8.2	+2.1	+4.4	+3.5	+2.5	−2.5	−1.4	−6.6	−5.2	−4.8	±4.1

From this table it is seen that the average error in the fifteen minute counts is 4.3 per cent., with a maximum error of 10 per cent. In the thirty minute counts, with the exception of a single smear, the average error is 3.8 per cent., and the maximum 8.7 per

[2] This experiment, of course, was not attempted till the technique had been mastered by preliminary work.

cent., the one exceptional smear giving an error of 24.6 per cent. In the sixty minute counts, the average error is 4.1 per cent., the maximum 8.2 per cent.

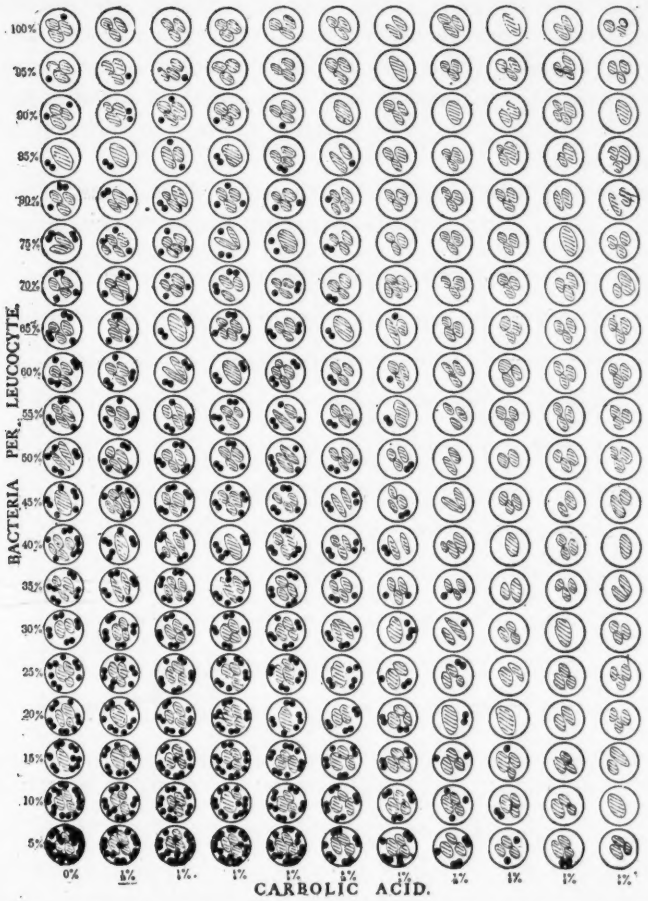

FIG. 1. Influence of carbolic acid on phagocytosis. Human leucocytes; average of 15, 30 and 60 minute counts.

In interpreting data obtained by the above technique, therefore, allowance must be made for a usual maximum error of about 10 per cent., but an occasional error (one count in thirty) of as much

as 25 per cent. must be expected. To eliminate this large experimental error, the average of a large number of data must be used.

Graphic Representation.—In recording data in most fields of quantitative chemistry, it is desirable to select a graphic method of representation. Such a method for the phenomenon of phagocytosis, must give not only the average number of the bacteria per leucocyte, and the number of leucocytes ingesting bacteria, but the numerical distribution of the bacteria among the different leucocytes, as well.

The graphic method selected is shown in the column "0%" of Fig. 1. This column consists of twenty diagrammatic leucocytes,

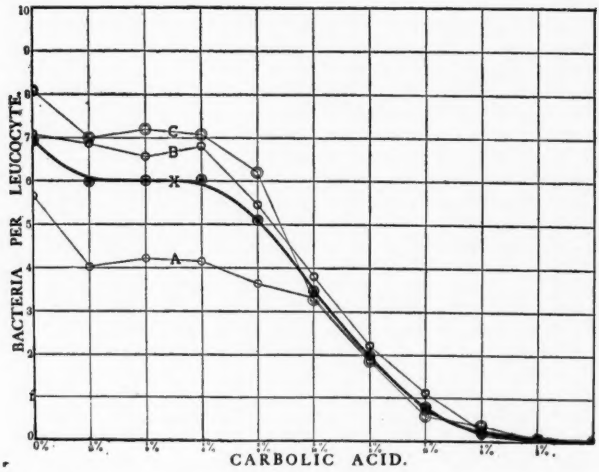

FIG. 2. Influence of carbolic acid on Wright's index. Data from Figure 1, $A = 15$-minute counts; $B = 30$-minute counts; $C = 60$-minute counts; $X =$ average.

each leucocyte representing 5 per cent. of the polymorphonuclear neutrophiles present in a given smear. In each leucocyte is marked the average number of bacteria found in its corresponding 5 per cent.

Carbolic Acid.—The average of the three counts obtained by the above technique, with carbolic acid, gives the data recorded in Fig. 1. These data can be translated in terms of the currently used

Wright's index, by calculating the average number of bacteria taken up per leucocyte. Such calculations are shown graphically by the broken lines *A, B* and *C* of Fig. 2. The average of these observa-

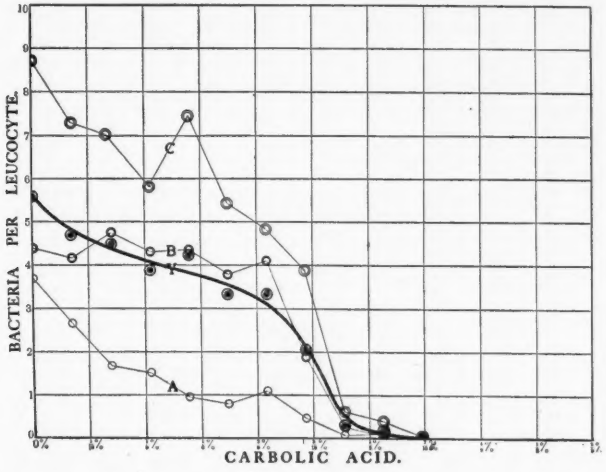

FIG. 3. Influence of carbolic acid on Wright's index. Rabbit leucocytes. *A, B* and *C* = 15-minute, 30-minute and 60-minute counts; *X* = average.

tions, plotted as smooth curves to eliminate experimental errors, as is customary in physical experimentation, is given in the heavy

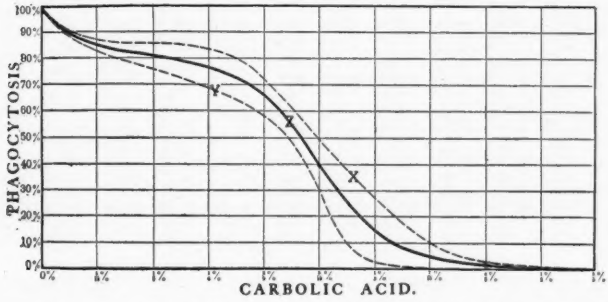

FIG. 4. Influence of carbolic acid on Wright's index; Curves *X* and *Y*, of Figs. 2 and 3, plotted to the same scale. *Z* = average.

curve *X*. A similar curve, obtained with rabbit blood in place of human blood, is given in Fig. 3. The curves of these two experi-

ments, reduced to percentage curves, by taking the phagocytosis in the control tubes of each experiment as 100 per cent., are shown in Fig. 4.

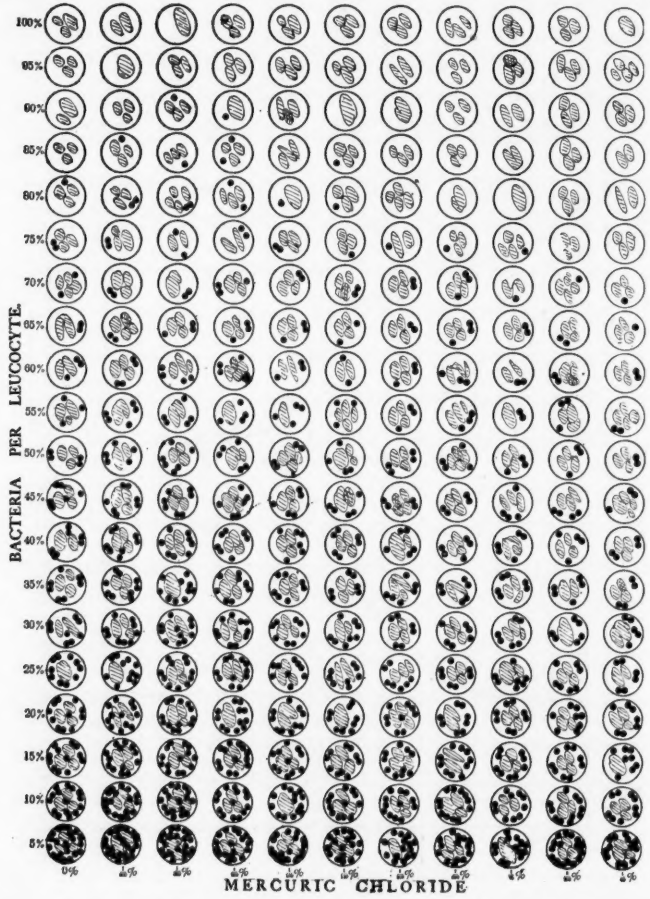

FIG. 5. Influence of mercuric chloride on phagocytosis. Human leucocytes; average of 15- and 30-minute counts.

From this figure, it is seen that the addition of carbolic acid to the experimental tubes causes, from the first, a decrease in phagocytic power, phagocytosis falling off about one third by the time the

carbolic acid reaches a concentration[3] of 2/9 per cent. A further increase in carbolic acid causes an almost precipitous fall in phagocytic power, phagocytosis being reduced seven eighths by the time

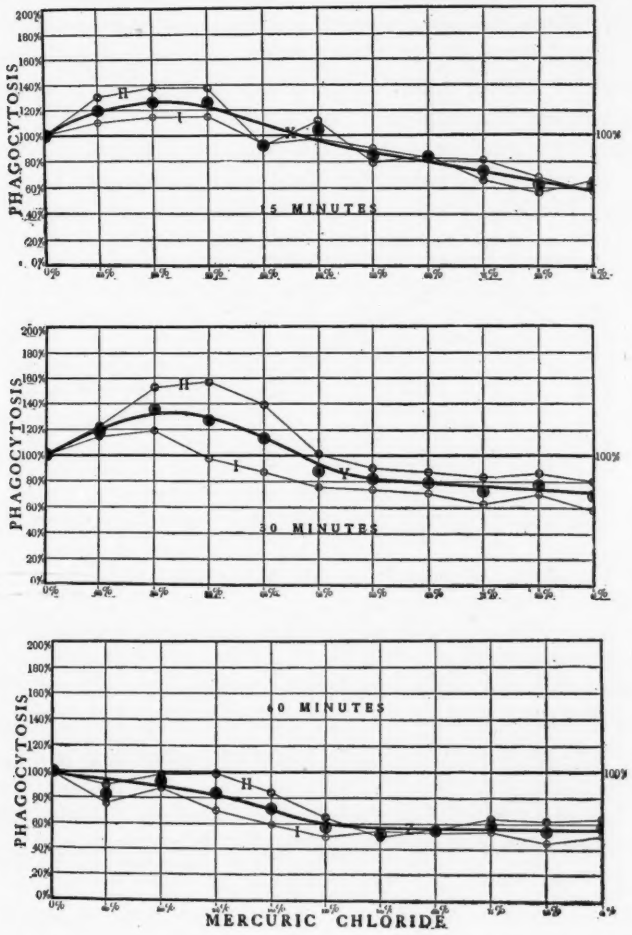

FIG. 6. Influence of mercuric chloride on Wright's index. 15, 30- and 60-minute data plotted separately. *I* = data from first experiment; *II* = data from second experiment; *X*, *Y* and *Z* = averages.

[3] Concentration measured in terms of final dilution.

the concentration reaches one third per cent., and entirely ceasing at one half per cent.

Mercuric Chloride.—The average of the fifteen and thirty minute counts, in two experiments with mercuric chloride are given in Fig. 5. Since the sixty minute counts and the earlier counts in these experiments show different effects of mercuric chloride, the influence of this antiseptic on phagocytosis can not be represented by a single curve, as was done with carbolic acid. Three percentage curves were therefore plotted, showing the changes in Wright's index, at the end of fifteen, thirty and sixty minutes. These curves are shown in Fig. 6. In each curve 100 per cent. stands for the

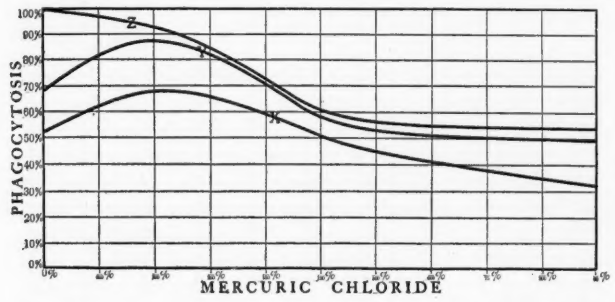

FIG. 7. Influence of mercuric chloride on Wright's index. Curves of Fig. 6 plotted to the same scale.

phagocytosis in the control tubes, containing no antiseptic.

These three curves may be reduced to the same scale by taking the normal sixty minute phagocytosis as 100 per cent. Thus reduced, they are shown in Fig. 7. From this figure, it is seen that mercuric chloride causes, in small amounts, a preliminary stimulation in phagocytosis, followed by a depression. The maximum preliminary stimulation is obtained with a bichloride concentration of 1/300 per cent. As the concentration increases beyond this amount, the preliminary stimulation decreases, and completely disappears at 1/120 per cent.

A further increase in mercuric chloride causes a pronounced fall in phagocytic power, early phagocytosis being reduced 50 per cent. by the time the concentration reaches 1/60 per cent. With con-

centration of 1/200 per cent. and over, phagocytosis practically ceases at the end of thirty minutes.

Boric Acid.—The average of the fifteen and thirty minute counts

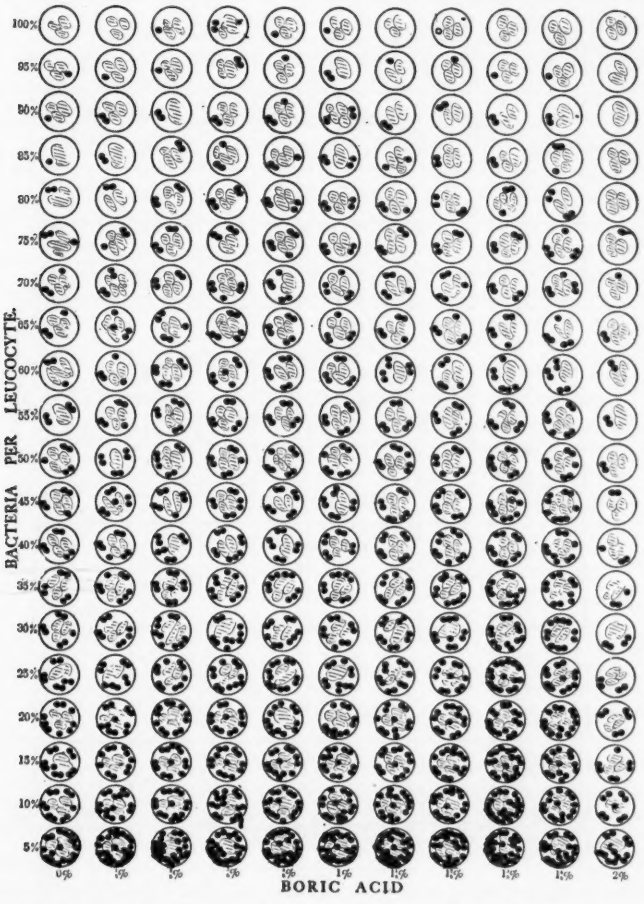

FIG. 8. Influence of boric acid on phagocytes. Human leucocytes, average of 15- and 30-minute counts.

in two experiments with boric acid are given in Fig. 8. With boric acid, as with mercuric chloride, the sixty minute counts

and the earlier counts show different effects on phagocytosis. Three percentage curves were therefore plotted (Fig. 9) showing the observed effect at the end of fifteen, thirty and sixty minutes. These curves are plotted to the same scale as given in Fig. 10.

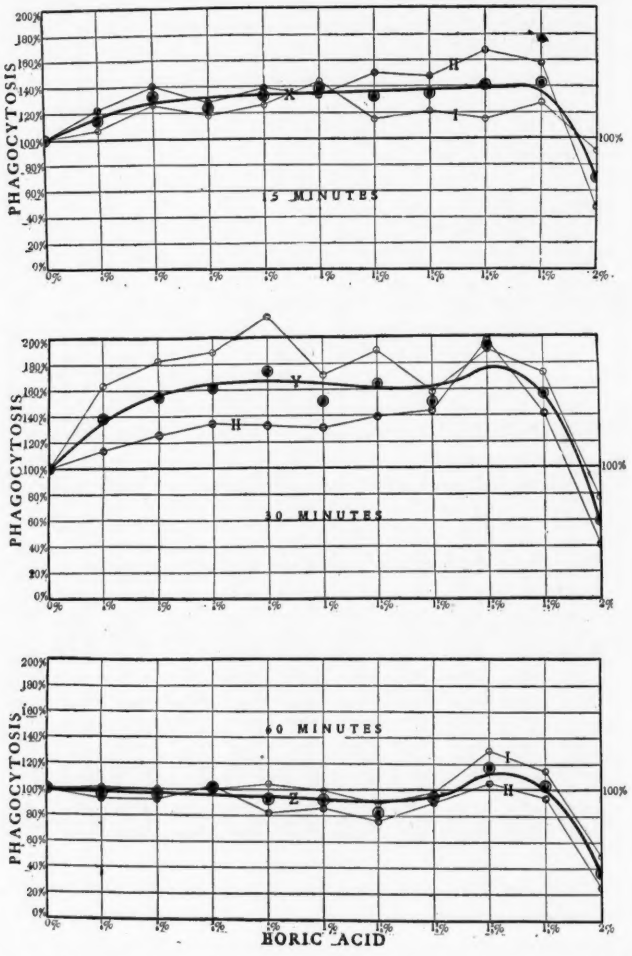

FIG. 9. Influence of boric acid on Wright's index. 15-, 30- and 60-minute data, plotted separately. *I* = data from first experiment, *II* = data from second experiment; *X*, *Y* and *Z* = averages.

From Fig. 10 it is seen that boric acid, in small amounts, causes a preliminary stimulation in phagocytosis, followed by a depression. This phenomenon becomes more marked as the percentage of boric acid increases, until a concentration of 1½ per cent. is reached. After this a further increase in boric acid causes a rapid fall in phagocytic power, phagocytosis apparently completely ceasing soon after the concentration reaches 2 per cent.

Quinine Hydrochloride.—The average of the three counts in an experiment with quinine hydrochloride are given in Fig. 11, and

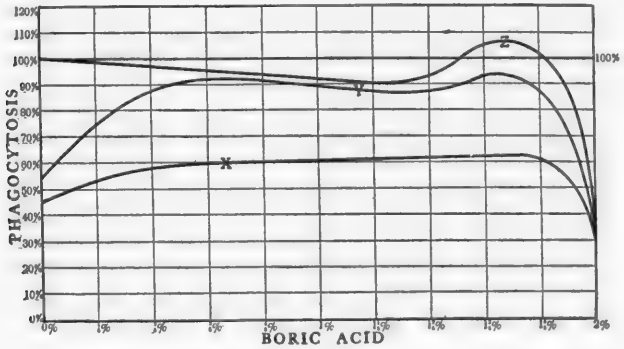

Fig. 10. Influence of boric acid on Wright's index. Curves from Fig. 9, plotted to the same scale.

the corresponding variations in Wright's index in Fig. 12. A duplicate experiment is given in Fig. 13, and the average of the two experiments in Fig. 14.

From this figure it is seen that the addition of quinine hydrochloride gives, from the first, an increase in phagocytosis, the increase reaching a maximum (20 per cent.) as soon as the concentration reaches 1/200 per cent. A further increase in quinine hydrochloride causes a decrease in phagocytosis, phagocytosis being reduced to normal by the time the concentration reaches 1/120 per cent., and apparently completely ceasing soon after the concentration reaches 1/40 per cent.

Whether the observed increase is in the nature of a permanent stimulation, or is only a temporary stimulation to be succeeded by a depression, has not been determined.

Summary.—Judging from counts made at the end of fifteen, thirty and sixty minutes the following influences on phagocytosis have been demonstrated:

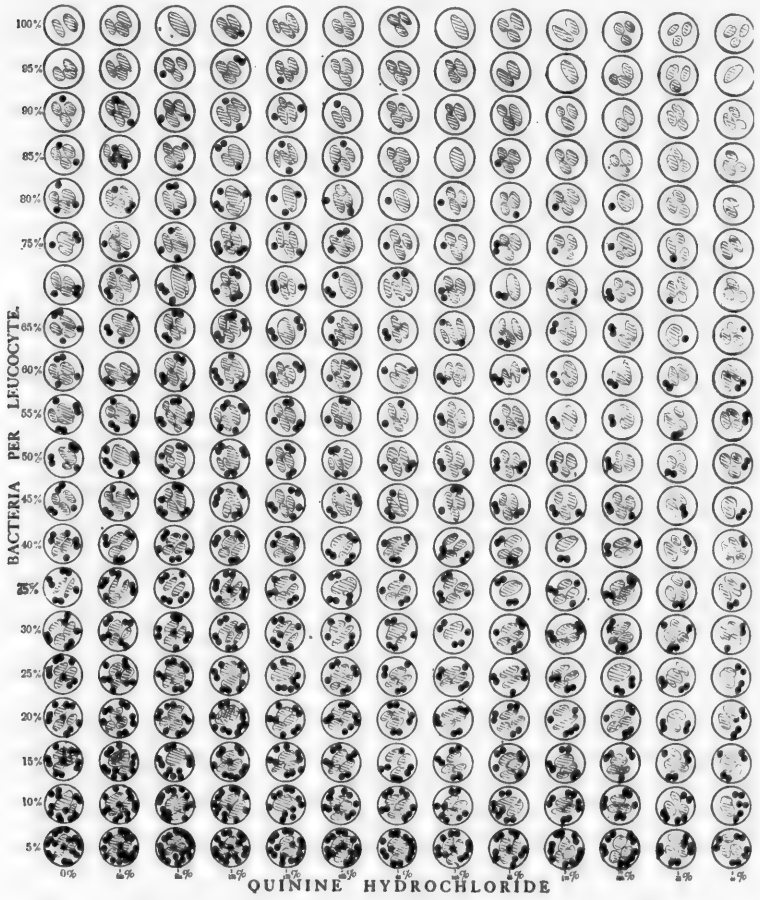

FIG. 11. Influence of quinine hydrochloride on phagocytosis. Human leucocytes; average of 15-, 30- and 60-minute counts; Experiment II.

1. Carbolic acid, added in increasing amounts to experimental tubes, causes from the first a decrease in phagocytic power, phagocytosis falling off one third by the time the concentration reaches

2/9 per cent., seven eighths by the time it reaches 1/3 per cent., and completely ceasing at ½ per cent.

2. Mercuric chloride, in concentrations less than 1/120 per cent.,

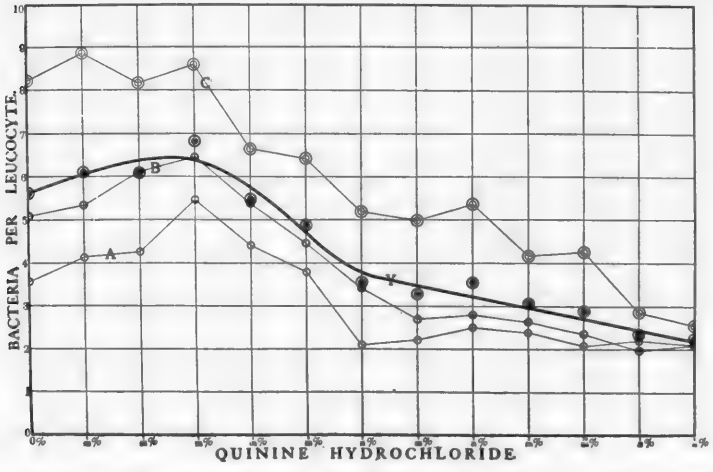

FIG. 12. Influence of quinine hydrochloride on Wright's index. Experiment II. *A*, *B* and *C* = 15-, 30- and 60-minute counts; *Y* = average.

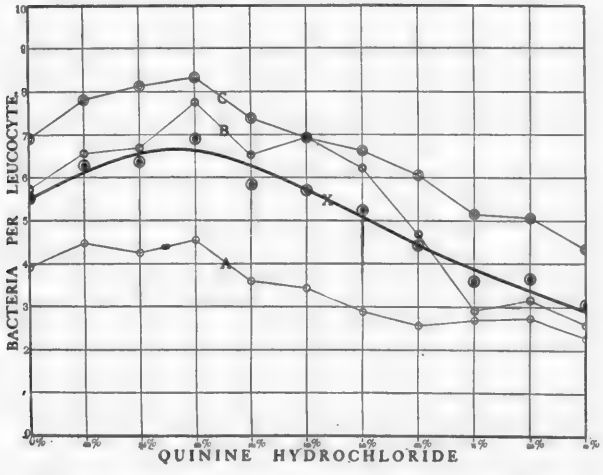

FIG. 13. Influence of quinine hydrochloride on Wright's index. Experiment I. *A*, *B* and *C* = 15-, 30- and 60-minute counts; *X* = average.

causes a transient stimulation in phagocytosis, followed by a depression. In larger amounts it causes a permanent depression from the first, phagocytosis apparently completely ceasing soon after the concentration reaches 1/60 per cent.

3. Boric acid, in concentrations less than 1½ per cent., causes a transient stimulation in phagocytosis, followed by a depression. As the concentration increases above 1½ per cent. there is a rapid fall in phagocytic power, phagocytosis apparently completely ceasing soon after the concentration reaches 2 per cent.

4. Quinine hydrochloride, added in increasing amounts, causes a

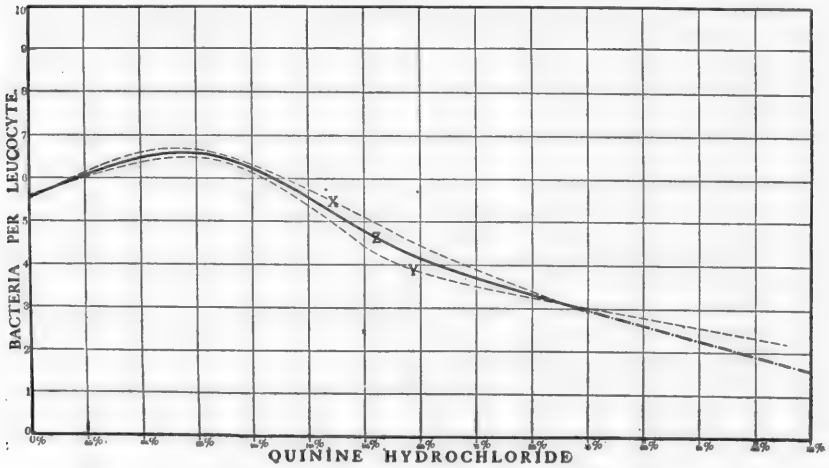

FIG. 14. Influence of quinine hydrochloride on Wright's index. Curves *X* and *Y*, from Figs. 12 and 13, plotted to the same scale; *Z* = average.

stimulation in phagocytosis, phagocytosis reaching a maximum as soon as the concentration reaches 1/200 per cent. A further increase in quinine hydrochloride causes a decrease in this stimulation, phagocytosis being reduced to normal as soon as the concentration reaches 1/120 per cent. In larger amounts quinine hydrochloride causes a depression in phagocytosis, phagocytosis apparently ceasing soon after the concentration reaches 1/40 per cent. Whether the observed stimulation is a permanent stimulation, or a transient stimulation to be succeeded by a depression has not been determined.

A FURTHER CONTRIBUTION TO THE KNOWLEDGE OF THE OPSONINS.[1]

By CHARLES E. SIMON, M.D., Baltimore.

(*From the Laboratory of Dr. Charles E. Simon.*)

In a previous communication I have drawn attention to certain fallacies which attach to Wright's method of estimating the opsonic content of the blood and have suggested that more accurate information may be obtained by diluting the blood in varying proportions and by determining the percentage of phagocyting cells in the resulting preparations. In order to obtain comparable results I proposed the use of maximal numbers of bacteria, as a matter of routine, so that deviations from the normal, in either direction, would be the more striking. The report of the work, in which I was assisted by Drs. R. V. Lamar and Bispham, was submitted to the Rockefeller Institute on the first of March, 1906. Its publication was unfortunately much delayed and it has thus far appeared only in part.[2]

Since that time Wright's work has attracted widespread attention and investigators generally have been following his methods. In the further prosecution of our own work it became necessary, for purposes of comparison, to follow Wright's technique at least in part, and above all to obtain an index which should be directly comparable to Wright's index.

After certain preliminary investigations I then suggested that by comparing the percentage of phagocyting leucocytes in the case of a specimen of blood under investigation with the figure corresponding to a specimen of pooled normal blood serum, terming the latter

[1] Conducted under a grant from the Rockefeller Institute for Medical Research. Received for publication June 20, 1907.

[2] Simon, C. E., and Lamar, V. R. A Method of Estimating the Opsonic Content of the Blood and Other Fluids. *Johns Hopkins Hospital Bull.*, 1906, xvii, 27. Simon, C. E., Lamar, R. V., and Bispham, W. N. A Contribution to the Study of the Opsonins. *Jour. of Exper. Med.*, 1906, viii, 651.

value 1, an index is obtained which is comparable directly to Wright's index. This index I have termed the "percentage index," as contrasted with Wright's "bacillary index." Working with Staphylococcus aureus emulsions prepared with special care and throwing out of the counts cells which had manifestly gotten into clumps (which after all not even with staphylococci can be altogether avoided) I could convince myself that the percentage index and the bacillary index may agree to the second decimal and that the results obtained with either method are directly comparable with each other. This fact having become established it was next ascertained that the percentage index could be used as a direct check upon the bacillary index. It was necessary, however, to work with fairly thin emulsions, viz., such as would furnish values of not much more than 50 per cent. for the normal control blood, in cases at least in which the bacillary index would suggest a material increase of the opsonic content. A normal value of 50 per cent. would allow for an increase of the patient's blood to 100 per cent., giving a maximal percentage index of 2. For most purposes this is sufficient; otherwise the blood must be progressively diluted, and the degree of phagocytosis in the diluted specimens compared with correspondingly diluted normal serum. With low values, on the other hand, the emulsions need not be so thin.

A few examples will illustrate the above points:

Example I.—The phagocytic index of the patient was 3.90 and of the normal control 4.88; the opsonic "bacillary" index was accordingly 0.79. The percentage of phagocytosis in the same patient was 60 and of the control 76 and the percentage index hence 0.78, showing the close correspondence of the two indices under favorable conditions.

Example II.—A patient's phagocytic index was 5.11 and that of the control 4.56; the opsonic bacillary index therefore was 1.09. The patient's percentage was 79 and that of the control 72, thus giving a percentage index of 1.09, showing a correspondence of the two indices to the second decimal.

While with especially good emulsions of staphylococci the two indices may thus agree very closely and at times actually coincide, deviations are frequently met with in routine work which show beyond all doubt that Wright's index, when not controlled by the percentage index, may give rise to erroneous conclusions; as a matter of fact results are at times obtained which are absolutely

absurd, and I have no doubt in the least that some of the phantastic curves which have been published are due entirely to errors of technique.

Example III.—The tuberculo-opsonic bacillary index in a case of suppurating bubo, in association with chancroid was 1.93, which according to Wright would have suggested a tubercular process with systemic manifestations. As a matter of fact there was no evidence whatever of any tubercular lesion and the percentage index was normal, viz. 1.18.

Sometimes an increase of both indices shows that there is actually an increased degree of phagocytosis, but the occasionally enormous difference between the two demonstrates that the higher bacillary index has been in part at least owing to the fact that leucocytes had been included in the count which had gotten into clumps and had thus artificially raised the phagocytic and therefore the opsonic index. This error could only have been avoided if in the normal control specimen a corresponding number of leucocytes had gotten into clumps of the same number of bacteria, which of course would have been purely a matter of accident. The suggestion naturally offers itself to exclude from the count cells which have manifestly gotten into clumps. The difficulty, however, would be to decide where to draw the line, which naturally would be more or less arbitrary. Then again it would be necessary to eliminate negative (empty) cells to counterbalance the discarded positive cells, and the question would arise in every case how many negative cells would have to be thrown out from the count for each positive cell, thus giving rise to further difficulties.

Example IV.—The staphylococcus percentage index in a case of sub-phrenic abscess was 1.46 and Wright's index 2.9, whereas dilution to 1:20 showed that there was no evidence of a material increase of the opsonins whatever, viz. 4 per cent., as compared with 8 per cent. on the part of the control.

It might of course be argued that since it is possible *at times* to obtain fairly homogeneous emulsions of organisms, this should be possible at all times, and that accordingly no control of Wright's index is necessary. Practically, however, this is not possible, even with staphylococci, and much less so with other organisms. It seems to me accordingly that a method with which reasonably

experienced laboratory workers cannot obtain uniform and corresponding results must either be abandoned, or it must in some measure be controlled or modified. My experience goes to show that the essential objection to Wright's method and the one which all "opsonic" workers realize is referable to the difficulty, if not the impossibility, of obtaining uniform emulsions. As I have said with staphylococci this is relatively small; with other organisms it is variable, and with the tubercle bacillus almost, if not entirely unsurmountable. Wright himself must realize this, as he has repeatedly changed his technique in this respect, and, it may be added, without announcing such changes, which after all are of fundamental importance. I have worked with extracted and non-extracted tubercle bacilli, with emulsions in 0.1 per cent. and 1.5 per cent. saline, but I have never seen an emulsion yet which was free from clumps.[3]

With other organisms still other difficulties enter into consideration. With the colon and typhoid bacilli, for example, there is usually such a profound degree of destruction with fragmentation and lysis of the organisms and coincident loss of reaction to stains even in normal blood, that a count of the organisms in the leucocytes must of necessity give rise to erroneous results. Even with staphylococci one meets with specimens every now and then, where the staining is very defective and may not be possible at all. I have seen cells filled with colorless "shells" of staphylococci, where a cursory examination would only have shown one or two stained organisms. Counting under such circumstances would have led to the most absurd conclusions, as is shown in the following table. The specimens were sent to me by Dr. North of the College of Physicians and Surgeons, New York, and were of the series which were being investigated by different laboratories for comparative purposes to ascertain the value of Wright's method. As normal control I used the pooled blood of four healthy individuals.

[3] Dr. Cole tells me that he is now working with tubercle bacilli which after cultivation on glycerine agar are killed off by exposure to sunlight, and that with such material he has obtained his best results. I have not yet had an opportunity to examine preparations made with such emulsions.

TABLE I.

Specimen.	Wright's Index.	Percentage Index.
1	.86	.51
2	1.11	.75
3	.81	.44
4	.90	.64
5	.88	.59
6	.88	.55
7	.90	.53
8	1.02	.52
9	1.02	.66
10	1.0	.59
11	.95	.58
12	1.11	.58

The correctness of my conclusions in reference to the accuracy of Wright's method will be borne out on examination by any one who will carefully compare the two indices. But I must insist that without the percentage method as a check upon the bacillary method the results obtained with the latter cannot be regarded as trustworthy.

During the past winter I had Dr. Knorr investigate this point in reference to the number of leucocytes that should be counted, and I append the results which were reached. The counts were made with emulsions of varying strength, and with normal blood serum. The results show that the percentage index fluctuates far less normally than the bacillary index. With emulsions containing from 666,000 to 2,000,000 organisms per cubic millimeter the percentage method yields fairly constant results, while with very thin emulsions it also becomes untrustworthy.

TABLE II.

A. *Emulsion of 2,000,000 cocci per c.mm.*

Cells Counted.	Wright's Index.	Percentage Index.
25	1.00	*1.26*
50	.97	1.00
75	.96	1.00
100	*1.69*	1.02
150	1.16	1.12
200	1.23	1.20
300	1.18	1.11
400	*1.27*	1.15

B. *Emulsion of 666,666 cocci per c.mm.*

25	*1.85*	1.18
50	*1.32*	1.16
75	1.03	1.00
100	*1.25*	1.02
150	*1.30*	1.02
200	1.12	.97
300	1.13	1.04
400	1.06	1.00

C. *Emulsion of 222,222 cocci per c.mm.*

25	1.20	*.60*
50	*1.62*	1.14
75	*1.75*	*1.27*
100	*1.75*	*1.26*
150	*2.21*	*1.60*
200	*2.15*	*1.70*
300	*2.00*	*1.62*

D. *Emulsion of 74,074 cocci per c.mm.*

25	*0.66*	1.00
50	*0.50*	*0.62*
75	*0.57*	*0.70*
100	1.14	1.26
150	1.18	1.15
200	0.95	0.96
300	1.05	1.11

As the normal variations in the opsonic index according to Wright may fluctuate between 0.80 and 1.20 it would follow, taking the entire series of examinations, as it stands, that Wright's method gave erroneous results in 50 per cent., while with the percentage method the percentage of error was 30. Throwing out the weaker emulsions we would have 37.5 per cent. of error for Wright's index and only 6.2 per cent. for the percentage index.

The data further show that even a count of one hundred or more cells is no safeguard against error, and that with the percentage index a count of fifty cells is sufficient.

The effect of the thickness of the emulsion upon the bacillary and percentage index is also shown in Table III, which at the same time illustrates how closely under favorable conditions the two will agree; it further emphasizes that the percentage index is a valuable check upon the other.

TABLE III.

No. of Cocci per c.mm.	No. of Cocci per Leucocyte.		Percentage of Phagocyting Cells in		Wright's Index.	Percentage Index.
	(a) Patient.	(b) Pool.	(a) Patient.	(b) Pool.		
3,750.000	2.54	3.15	87	82	.80	.94
1,865.000	1.68	1.90	68	70	.88	.85
937.500	1.26	1.36	58	65	.90	.89
468.750	1.02	1.78	50	45	1.30	1.11
234.75	.73	.71	37	33	1.02	1.05

Working with the two indices side by side during the past winter, I found, as I have said before, that under favorable conditions the two may coincide. If an increase or decrease of Wright's index is associated with an increase or decrease of the percentage index, it may be assumed that such an increase or decrease is not due to errors of technique, and may be interpreted as actually expressing what Wright's index can express. If, on the other hand, the one goes up and the other down, experience has taught me that in such cases the percentage index should be accepted in lieu of the other.

It will be noted that I have just used the expression "what Wright's index *can* express." I do so advisedly, for I am still of the opinion elaborated in a previous paper, that Wright's method does not furnish a proper index of the quantity of opsonic material which is actually present, and I still insist that the dilution method gives results which are more in accordance with the actual facts. I have pointed out that conditions may occur in which the opsonic content of two specimens of concentrated blood serum is apparently the same, but that on progressive dilution the one rapidly loses its opsonifying power, while the other may retain it to a marked extent. This may be observed not only under pathological conditions but at times also in perfect health. I have given examples in one of my previous communications and Amberg[4] has brought out the same point in his studies on the opsonic content of the blood of infants.

It may be objected that in my previous studies emulsions of constant bacterial content were not used, but this, I think, is invalidated by the fact that we always used maximal amounts, and that as a consequence variations from the normal are brought out into

[4] Amberg, *Jour. of Amer. Med. Assoc.*, 1907, xlviii, 304.

bolder relief. After my second winter's work I feel as confident, as I did a year ago, that Wright's method does not furnish a proper index of the opsonic content of the blood, and I am aware of the fact that many of those who have worked upon the same problem have reached the same conclusion. I am not willing, however, to discard the principle of the matter, but would urge other opsonic workers, who stand ready to abandon Wright's opsonic index, to resume their work once more, using my method of dilution. The avowed purpose of taking the opsonic index, aside from diagnostic purposes, is the idea of having a guide to dosage and frequency of inoculation in the treatment of diseases by means of bacterial vaccines. All those who have busied themselves with this subject, and have honestly and without preconceived ideas investigated it, are no doubt ready to admit that the introduction of the bacterial vaccines marks an important epoch in the advance of rational therapeutics. The point at issue at present is the question whether Wright's opsonic index furnishes the desired guide to dosage or not. I, for one, am convinced, as the result of my own work, that in the majority of cases which Wright has suggested as suitable for vaccine treatment, and using the small doses which he has advised, at intervals of one or two weeks, such treatment can be carried out without detriment to the patient, without the use of any index. I have never seen any " negative " phases under such conditions, which could not be perfectly well explained as being due to unavoidable errors of technique. If, however, the attempt at immunization be pushed by materially increasing the frequency of the injections, or by using large doses, I feel that harm may be done and that in such cases even so coarse a guide as the index is better than none. To be sure the physical signs and the patient's general condition may furnish some indication whether the dosage has been too high, but I am a little doubtful in my mind, whether it is desirable to let matters come to such a point, where the physical signs and the general condition of the patient tell us that we have done harm, even though this be but temporary. The only " negative " phases that I have seen have occurred under such conditions, and I feel confident that the result could have been avoided had lower doses been used first and controlled by the index.

The following case will serve to illustrate this point.

A little boy of eight, the subject of recurrent epulis, was injected with 575 millions of Staphylococcus aureus on Nov. 20th; his index on the 13th had been .62 (bacillary) and .80 (percentage); on the 15th it was 1.4 (bacillary) and 1.0 (percentage), and on the day of the injection 1.6 (bacillary) and 1.1 (percentage). During the night of the 20th to the 21st his temperature rose to 103°, followed by a profuse sweat, and on the 27th the index had dropped to .30 (bacillary) or .45 (percentage). By December 4th the two indices had risen again to .80 and .90 respectively, and on the 11th they were 1.0 and .80. On that day an injection of 600 millions was given, which was followed by a drop to .10 and .22 respectively (see Curve I).

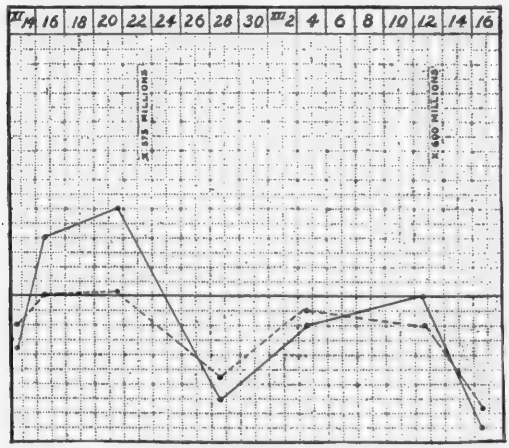

FIG. 1. Solid line indicates the bacillary index; dotted line indicates the percentage index.

Drops in the opsonic index such as these, following upon the injection of bacterial vaccines, occur very rarely in my experience, if small doses are used. They do occur, however, and I personally have the conviction, that it cannot be a matter of indifference to the patient, if his phagocytic power is practically brought to nil, and especially so in those infections in which the process of phagocytosis is one of the main stays of resistance.

During the past winter I have had occasion repeatedly to use bacterial vaccines in *acute* staphylococcus infections, and I may add, at times with very good results. The clinical course in these cases made it clear that injections given a week apart would have been without avail, and an attempt was therefore made to stimulate

the mechanism of immunization by pushing the vaccine. The question naturally was what guide to dosage should be used, and, in lieu of a better, the opsonic index was followed. These cases will be reported in detail at another occasion, but it may not be out of place to cite at least one.

The patient was a young women of about twenty-five, who a week after childbirth was seized with a chill with a temperature elevation to 104.1°; the next day she had another chill with a temperature of 104°. From this day her temperature varied between 101° and 105°. I saw her for the first time on the evening of Jan. 21st, eight days after the onset of the fever. At that time the temperature was 104.8°; she was quite somnolent and made the impression of a very sick woman. The physician in attendance had isolated Staphylococcus aureus, and she was accordingly injected with aureus vaccine. One thousand millions were given on the evening of the 21st and another dose the following morning. Her temperature promptly came to normal and remained so until the 26th. On that day and the following there was an evening rise to 102.5°. She received 750 million cocci on the evening of the 27th, and the same dose on the morning of the 28th. Her temperature promptly again came to normal and remained so. Her index (percentage) at the time of the first injection was .95; the next morning 1.0 (no negative phase) and on the 23d, viz. 36 hours after the first and 24 hours after the second injection, 1.90. The further course is seen in the curve (Curve II).

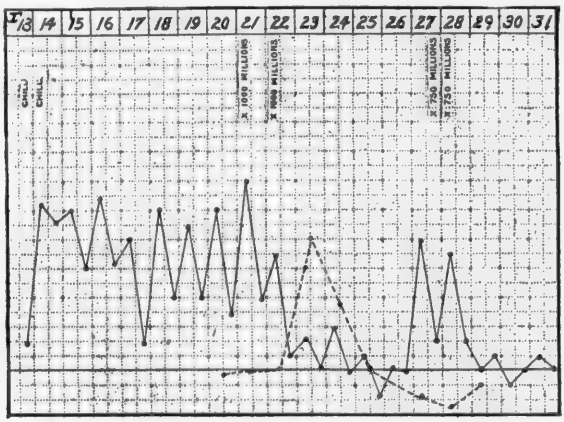

FIG. 2. Solid line indicates highest and lowest temperatures of the day; dotted line is the opsonic curve.

I have been surprised to see to what extent the injection of the staphylococcus vaccines can be carried in some cases, and manifestly not to the detriment of the patient. In one of my acute

cases 1,000,000,000,000 were injected within a week, and I may add without the appearance of a negative phase, and with an excellent effect upon the patient's condition (staphylococcus septicæmia with multiple abscesses). Nevertheless I hardly feel willing to carry the injections to such a point without some guide, better than the physical signs and the patient's general condition, and I feel convinced that the opsonic index in such cases is of service. But I repeat once more *with small doses,* such as Wright recommends, *administered at intervals of a week or two in chronic, well localized infections the opsonic index is of little, if any real value, either as a guide to dosage or to the time and frequency of inoculation.*

THE SPECIFICITY OF THE OPSONINS.

As regards the specificity of the opsonins my earlier investigations led to the conclusion that under normal conditions at least a specificity of the opsonins does not exist,[5] and I suggested then that it is quite conceivable that as a result of infection, viz., immunization substances may be produced either *de novo,* or increased in amount, if preëxistent, which may so influence a given organism that under the subsequent action of a common non-specific opsonin, it is taken up in larger or smaller numbers, as the case may be.

During the past winter I have repeated many of the experiments of the previous year, to eliminate possible errors of technique, but have been forced to the same conclusion, viz., that *the opsonins of normal blood serum are non-specific.*

This conclusion is based upon absorption experiments which were analogous to those previously reported. Since some of the organisms, however, and notably when relatively avirulent, are taken up by the leucocytes already in physiological salt solution (Bacillus subtilis, Bacillus pyocyaneus, Bacillus coli), and since some of Bulloch and Western's[6] contrary results may possibly be explained upon this basis, I chose only such organisms which had recently been isolated from infected individuals. Working with these I could find no evidence whatever that absorption of the opsonins for one organism leaves opsonins for other organisms behind. To be

[5] *Jour. of Exper. Med.* 1906, viii, 651.

[6] Bulloch, W. and Western, G. J., *Proc. Royal Soc.,* 1906, lxxvii, 531.

sure I never carried the absorption to a point where opsonic action ceased altogether, but in all cases there was a marked drop for both.

Example I.—About 0.5 c.c. of normal blood serum was inoculated with one-half of a twenty-four hour staphylococcus agar culture, and incubated for twenty minutes at 37° C. The percentage of phagocytosis before inoculation was 100 for the staphylococcus and 90 for the colon bacillus. After incubation the serum was centrifugalized at high speed for one hour, the supernatant fluid transferred to a new tube and centrifugalized for an additional half hour. The percentage of phagocytosis, for the same emulsions, was then 60 for the staphylococcus and 50 for the colon bacillus.

Example II.—In another experiment the values before absorption were 64 for staphylococcus and 96 for colon, and 44 and 40 respectively after absorption with the staphylococcus.

Example III.—In this instance the staphylococcus value before inoculation with staphylococci was 96 and the colon value 64, while at the end of the experiment they were 16 and 20 respectively.

While my results thus do not agree with those of Bulloch and Western it is possible, as I have pointed out, that the phagocytosis which they noted for the non-absorbing organism after absorption with the other, may have been due to non-virulence of the bacteria used and consequent spontaneous phagocytosis. In one class of his experiments Bulloch uses Bacillus pyocyaneus after absorption of the serum with staphylococci, and finds that the serum has lost its opsonic power for the staphylococcus, while that for the pyocyaneus is preserved. This is not at all surprising since laboratory cultures of the pyocyaneus are taken up very readily in physiological salt solution.

I have not used the tubercle bacillus in my experiments, as living virulent organisms were not available.

Bulloch's observations that inoculation of a human being with tuberculin or with staphylococcus vaccine causes a quantitative increase in the tuberculo- and staphylococcus opsonins respectively, while the staphylococcus and tubercle opsonins in turn remain unaltered, are not well adapted to either prove or disprove the point at issue. As a result of the injection of bacterial vaccines the entire immunization mechanism of the body is thrown into activity and immune opsonins possibly formed at the same time. So long as the identity of normal and "immune" opsonins, however, has not been proven it seems to me that conclusions based upon the for-

mation of the latter are not applicable in the discussion of the specificity of the opsonins of normal serum. I should like to remark nevertheless that the injection of either tubercle or staphylococcus vaccine in the small doses recommended by Wright, does not necessarily cause an increase in the opsonic index in reference either to the one organism or the other.

Example I.—Mr. C. medical student. Staphylococcus index on Jan. 29th was .90. He was injected with 750 millions of staphylococci on Jan. 30th and on Dec. 2d the index was 1.15.

Example II.—Mr. R. Staphylococcus index on Jan. 10th .95; injection of 700 million staphylococci; index on the 12th 1.04.

Example III.—Miss R. Tubercle index on Jan. 29th .83; injection of 1/1000 mgrm. of tuberculin on the 30th; index on Feb. 2d .90.

These observations thus do not tend to bear out the correctness of Bulloch's view that the opsonins of normal serum are specific and my experiments on the blood of different animals, not only of different species but of the different classes of vertebrates in general, in all of which I could demonstrate the presence of opsonins for various organisms, similarly point to the same conclusion. Particularly striking is the fact that in the lower classes of vertebrates I could demonstrate the presence of opsonins for organisms with which infection does not occur in these animals. I recall the marked opsonifying power of the blood of the terrapin, of the frog and of fowls for Staphylococcus citreus, as an example.

Passing from the consideration of the specificity of the opsonins in normal blood to that of the blood in the infected individual there is evidence to show that here actual specificity may exist, but I must add, does not necessarily exist. While under normal conditions the opsonic content of the blood varies within relatively narrow limits, the variations in infections are much wider. These limits, so far as the "index" goes, Wright and his coworkers have placed at 0.8 and 1.2. My inclination would be to extend them somewhat more, for I have found indices as low as 0.70 and as high as 1.25 in individuals who showed no evidence whatever of any infection. At the same time I must admit that this is exceptional. This question is an important one in deciding from how many individuals blood should be taken to make up a normal control serum. I had Dr. Knorr investigate this point with the result

shown in the accompanying table. Dr. Knorr's blood served as patient's blood and was controlled by the pooled blood of an increasing number of supposedly normal individuals. Dr. K.'s phagocytic index for staphylococcus emulsion was 1.14; the percentage of phagocytosis was 56.

TABLE IV.

No. of Persons Furnishing Control Blood.	Per Cent. of Phagocyting Cells.	Phagocytic Index.	Bacillary Index.	Percentage Index.
1	62	1.24	0.91	0.90
2	57	1.18	0.96	0.98
3	60	1.42	0.80	0.93
4	56	1.16	0.98	1.00
5	64	1.19	0.95	0.87
6	71	1.52	0.75	0.79
7	58	1.11	1.00	0.96
8	60	1.40	0.81	0.93
9	68	1.48	0.77	0.82
10	71	1.52	0.75	0.79

From this table it is clear that it is usually not necessary to make up a pooled serum from many individuals, and for routine work the serum from one normal person is really sufficient, providing that this has been previously and repeatedly tested to exclude the possibility of its being normally either unusually high or unusually low. The serum of women during the menstrual period cannot be used as control serum, as the index during that time is abnormally low (see Table V).

TABLE V.

	Percentage Index.
A	0.57
B	0.54
C	0.53
D	0.24
E	0.72
F	0.54

As the lowered index does not affect one single species of organisms, but seems to be general, this fact also might be adduced as further supporting the view of the non-specificity of the normal opsonins at least. It is quite conceivable, considering the more or less uniform occurrence of constitutional disturbance at that time, that substances may be circulating in the blood which produce an inhibitory effect upon the opsonins.

While then the opsonic index in normal individuals (barring women during the menstrual period, and the exceptional individuals of whom I have spoken) varies within comparatively narrow limits, most remarkable deviations from the normal figure may be met with in disease. In their earlier work Wright and Douglas[7] reported a series of staphylococcus infections (furunculosis, sycosis, acne, etc.) in all of which the opsonic index was abnormally low, ranging from 0.1 to 0.87. They add that they have not come across any instance of the association of a normal phagocytic power with a staphylococcus infection. Analogous results were obtained in cases of tuberculosis. In one series of seventeen cases the values ranged between 0.4 and 0.85, including cases of tubercular peritonitis, laryngeal phthisis, psoas abscess, lupus, pulmonary phthisis, etc. In a series of twenty-five cases of pulmonary tuberculosis other than the acute form, Lawson and Stewart[8] also found low figures, on the whole, although in some the values were but little, if at all, below the normal average, viz., 0.5 to 1.0.

Subsequently Wright pointed out that low values are the rule in strictly localized infections, while in systemic infections abnormally high values may be observed.

As the index in the various infections shows deviations from the normal which usually only affect the offending organism, and as the injection of bacterial vaccines in such cases has a direct effect upon the corresponding bacterial index, Wright and his colaborers conclude that these facts indicate that the opsonins in the infected organism are specific. As they do not admit that a material difference exists between the opsonins of normal serum and the serum of infected individuals they further conclude that the normal opsonins also are specific. I have pointed out that there is no satisfactory evidence to support this view.

In studying the question of the specificity of the opsonins in the infected organism, which we shall refer to for the present, as a matter of convenience, as "immune opsonins," a series of absorption experiments were undertaken which were perfectly analogous to those described before, but in which the serum of infected indi-

[7] Wright and Douglas, *Proc. Royal Soc.*, 1904–5, lxxiv, 147.
[8] Lawson and Stewart, *Lancet*, 1905, ii, 1679.

viduals was exhausted, viz., of persons and animals which had been treated with bacterial vaccines. My experiments thus far have reference largely to staphylococcus and typhoid infections, but in neither could I find any evidence that absorption with one organism removed a homologous opsonin and left a heterologous opsonin behind. Working with dried and pulverized tubercle bacilli, on the one hand, and staphylococci, on the other, I have obtained results which corresponded in a way to those of Bulloch, viz., after absorption with staphylococci I occasionally found a slightly higher value for the tubercle bacillus than for the staphylococcus. If the experiment was reversed, however, the tubercle value was still higher. This leads me to think that the causes of the higher retention lay in the powdered bacilli and not in the serum, and that a different result might have been reached, if live virulent tubercle bacilli had been used instead. But, as I have said, such were not available at the time. As far as they go, however, my absorption experiments do not bear out the existence of specific "immune" opsonins.

A further argument which has been advanced as supporting the idea of the existence of specific opsonins, has reference to the greater thermolability of the normal opsonins as contrasted with the thermolability of the opsonins of "immune" sera. Wright, to be sure, does not admit that a material difference exists between the two, for he says:[9] "Manifestly the plain teaching of our experiments is, that the opsonin which is found in the heated immune serum of a patient who has responded to tubercular infection (I assume that his remarks would apply to any other infection in which phagocytosis plays a *rôle*), or as the case may be to the inoculation of a tubercle vaccine, does not differ, with respect to its resistance to heat and sunlight, from the opsonin which is found in the unheated normal serum. A precisely similar conclusion with respect to the identity of the opsonins found respectively in unheated normal and heated immune sera. . . ."

Other observers do not agree with Wright in his assumption that the opsonins of normal and immune sera are identical, notwithstanding his attempted explanation to this end. He himself, how-

[9] *Proc. Royal Soc.*, 1906, lxxvii, 224.

ever, manifestly attaches special significance to the fact that the opsonins of immune sera may be thermostable, or, as it would possibly be better to say, that they contain a thermostable component. The fact that he suggests that the thermostability of the opsonin toward a single organism may be used for diagnostic purposes shows that in his mind this thermostability is intimately associated with the question of specificity. In two tables he gives examples illustrating the thermostability of the opsonins toward the tubercle bacillus and introduces them with the remark that they represent a typical selection from a very extensive body of observations which furnishes the basis for the preceding statement as follows: "When a serum is found to retain in any considerable measure, after it has been heated to 60° for ten minutes, its power of inciting phagocytosis, we may conclude that 'incitor elements' have been elaborated in the organism, either in response to autoinoculations occurring spontaneously in the course of tubercular infection, or as the case may be, under the artificial stimulus supplied by the inoculation of tubercle vaccine." This is important, as Wright himself thus restricts the drawing of any diagnostic inferences from absence of thermostability. It is remarkable, however, on the one hand, that many cases do occur in whom there is every evidence of systemic infection, without thermostability of the opsonins, and that thermostable opsonins, on the other hand, may be found in persons in whom there is no evidence of infection.

TABLE VI.

Showing a series of cases in which active phagocytosis was not obtained after heating the sera for 10 minutes to 60° C.

Number.	Nature of Infection.	Unheated Bacillary Index.	Serum Percentage Index.	Heated Bacil. Index.	Serum Percent. Index.
Miss R.	Vesical tuberculosis.	.57	.66	0	0
Mr. G.	Tuberculosis of cæcum.	1.10	.91	.08	.03
Mr. G.	Tuberculosis of cæcum.	.95	1.08	.17	.25
Mr. B.	Renal tuberculosis.	.75	.80	0	0
Miss M.	Tubercular conjunctivitis.	—	.81	—	.09
R.	Staphylococcus suppuration.	.53	.94	.03	.20
Miss Sch.	Staphylococcus septicæmia.	.08	.27	.03	.11
Miss Sch.	Staphylococcus septicæmia.	2.36	1.53	.08	.26
G.	Abscess of finger (staph.)	1.63	1.26	.06	.06
Z.	Drained pelvic abscess (staph.)	1.36	1.20	.06	.06
M. D.	Tubercular hipjoint.	1.2	.9	0	0
X.	Tubercular elbow joint.	—	.85	—	0

Of cases in which marked thermostability was observed without any evidence of infection of any kind I may mention two. In the first there was from time to time hæmaturia of slight grade without any apparent cause. The patient was examined in great detail by Dr. H. A. Kelly, with negative result; tubercle bacilli could not be demonstrated in the urine; an injected guinea-pig remained well and the tuberculin test was negative. The tuberculo-opsonic index on three successive days varied between .52 and 1.20; corresponding to the latter value the index with the heated serum was .39.

In the second case there was likewise no evidence of tuberculosis with the usual methods of examination; the tuberculo-opsonic index, however, varied between .36 and 1.6 with the unheated serum and between .26 and .45 with the heated.

In studying these negative cases and contrasting them with my positive cases and with those of Wright, I cannot help but feel that in some of the positive cases the reaction in question is specific, but that in the negative cases we are dealing with normal non-specific opsonins. The difficulty may lie in our present inability to differentiate between infection *per se* without immunity production and infection associated with immunity production. This is even more so the case when we study the opsonic index toward a given microorganism from the standpoint of differential diagnosis, aside from the question of thermostability. Wright's conclusions that deviations from the normal values may be interpreted as evidence of infection with the homologous organisms suggest very strongly that a specificity exists, but it is difficult to understand, on the other hand, why so many cases of infection do not show any material deviation from the normal and cases, moreover, in whom there could be no doubt that systemic reaction was going on. Table VII shows a series of such cases.

In one case of acute staphylococcus septicæmia which ended fatally on December 15 and in which daily observations had been made since December 8, an abnormal index was only noted on two occasions, viz., .40 on December 8 and 2.0 on the twelfth; on the remaining days the values were entirely normal.

While my observations bear out the correctness of Wright's conclusions in a general way, that deviations from the normal for a

TABLE VII.

Showing a series of cases of both localized and systemic infections in which no material deviation from the normal-homologous index was obtained.

Name.	Disease.	Percentage Index.
Miss W.	Acne	1.0
Mr. B.	Renal tuberculosis	1.25
Mr. G.	Tuberculosis of the cæcum	1.25
L. B.	Glandular tuberculosis undergoing treatment with with tubercle vaccine	.90
S.	Tubercular hipjoint	1.10
J.	Tubercular hipjoint	.87
C.	Suppuration following operation	1.00
Dr. S.	Furunculosis	.75
Sp.	Suppuration following burns	.93
R.	Suppuration following operation	.94
W.	Staphylococcus abscess of neck	.88
J.	Suppurating bubo	.81
W.	Tubercular adenitis	1.18
F.	Staphylococcus septicæmia (multiple abscesses)	.90
L.	Staphylococcus septicæmia	.95

given organism may be regarded as important evidence from the standpoint of differential diagnosis, normal values can manifestly not be interpreted as excluding the possibility of infection with the organism under consideration.

But, as I have just said, my conclusion in reference to deviations from the normal coincide with those of Wright, only in a general way, for I have seen marked deviations both upward and downward for various organisms in cases in which there was not a corresponding infection; and the fact, moreover, that in some of these cases, at any rate, the deviations had reference not to a single species of organism seems to me to be further evidence to show that the opsonins in question were not specific.

One case in point (Mason) was one of syphilis of the liver with ascites; there was no evidence whatever of tuberculosis, while the tuberculo-opsonic index was .27.

In a second case of hepatic syphilis with ascites both the tuberculo-opsonic index and the staphylococcus index were increased, 1.4 in the case of the former and 1.3 in the latter (the bacillary indices were 2.6 and 2.3 respectively).

In a case of sarcoma of the breast and the axillary glands the tubercle index was .40 and the staphylococcus index .73 (the bacillary indices .39 and .37).

A typhoid patient in the second week of the disease gave a staphylococcus index of 1.46 (1.85 bacillary), in the absence of any apparent staphylococcus infection.

I further append some staphylococcus citreus values, obtained with my method of dilution, in cases in which a citreus infection could hardly be assumed to have existed.

TABLE VIII.

Disease.	1:20 Dilution.	1:30 Dilution.	1:40 Dilution.
Normal (average).	37.2	19.4	9.2
Appendicitis.[10]	96	88	—
Myelogenous leukæmia.[11]	—	6	0
Syphilitic ulcer of cæcum.	18	0	0
Carcinoma (generalized).	0	3	0
Appendicitis.	96	92	80
Retroperitoneal sarcoma.	—	68	64
Syphilitic cirrhosis.	100	96	—
Pneumonia.	100	92	88
Pneumonia.	100	88	84
General carcinomatosis.	100	92	64
Appendicitis.	100	100	100
Pneumonia.	100	88	60
Appendicitis.	20	—	0
Beri-beri.	4	0	0
Ruptured extra-uterine pregnancy.	12	4	0
Melanotic sarcoma.	8	—	0
Pernicious anæmia.	92	42	28
Spleno-medullary pseudo-leukæmia.	88	60	20

With these various facts before us, I believe, the conclusion is justifiable that *granting the opsonins to be definite entities their specificity, even in cases of infection has not yet been established, although certain data seem to point in that direction.*

OPSONIC IMMUNITY.

From the writings of Wright the majority of his readers no doubt have gained the impression that as a consequence of successive inoculations of bacterial vaccines an opsonic immunity gradually becomes established, which would be characterized by a persistingly high index, a continuous "high tide phase" to use Wright's own expression. Unfortunately there are no exact data published by Wright to throw light upon this question. My own investiga-

[10] I have included appendicitis cases in this table, since staphylococci are only very rarely found under these conditions and there is little reason to assume that they were staphylococcus infections.

[11] This low value was obtained during a period of great improvement in the patient's condition with practically normal blood picture.

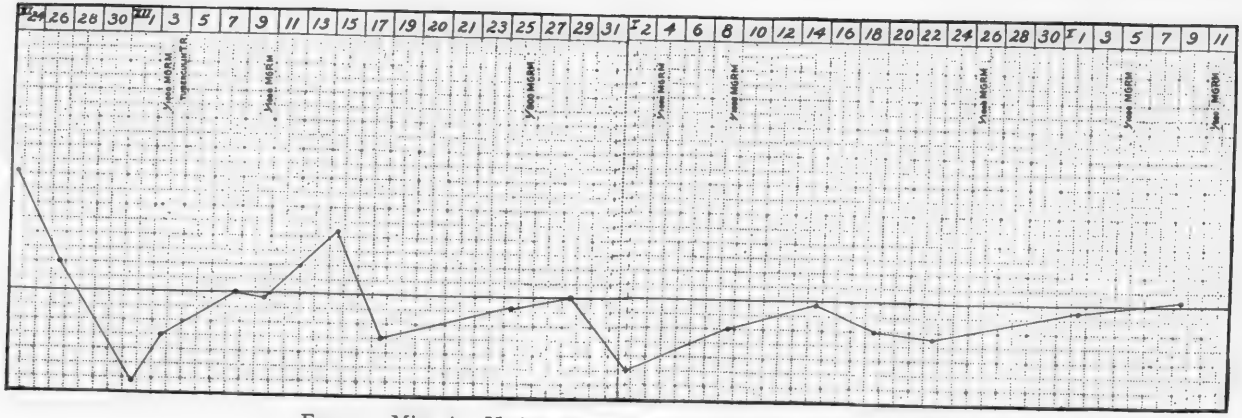

FIG. 3. Miss A. Vesical tuberculosis; recovery under treatment.

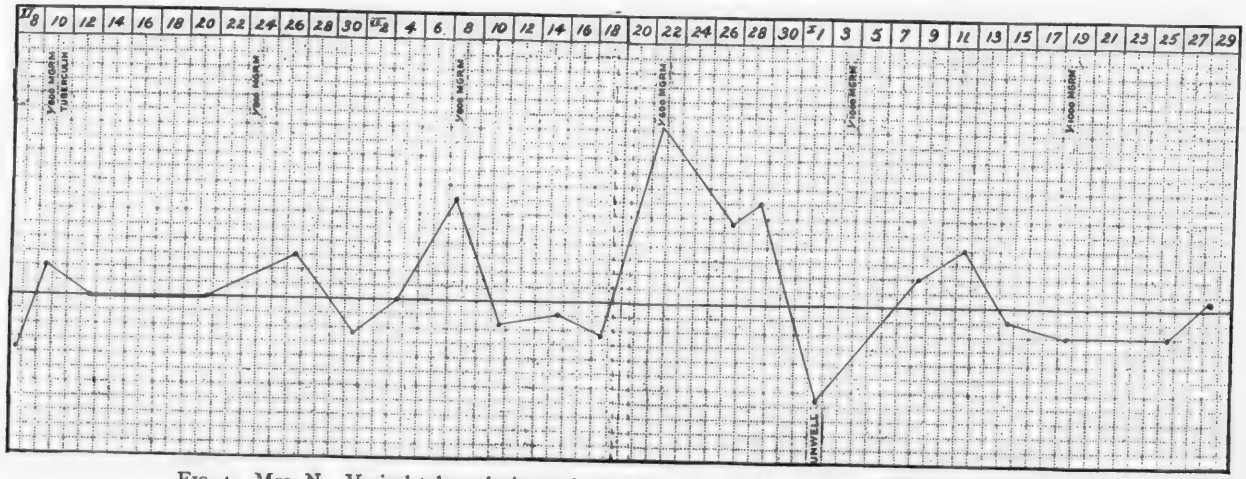

FIG. 4. Mrs. N. Vesical tuberculosis; under treatment nearly a year with great improvement.

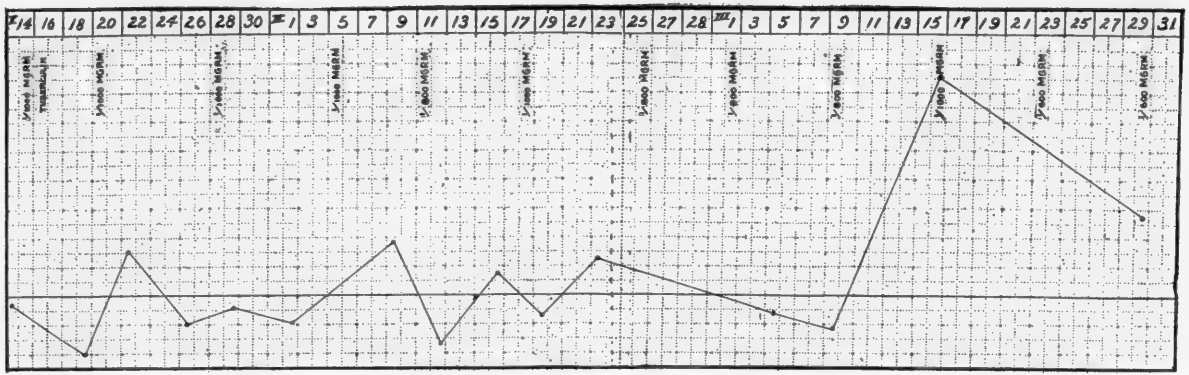

FIG. 5. Mr. B. Tubercular horse-shoe kidney and calculous pyelitis; gain in weight under treatment, but tubercle bacilli continue in the urine.

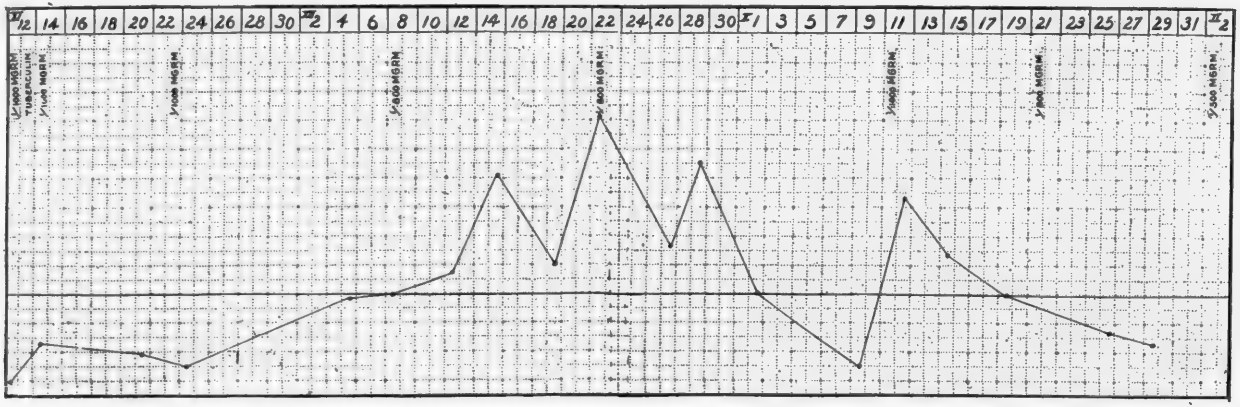

FIG. 6. Miss H. Tubercular peritonitis; operation; tuberculin treatment; recovery after severe illness.

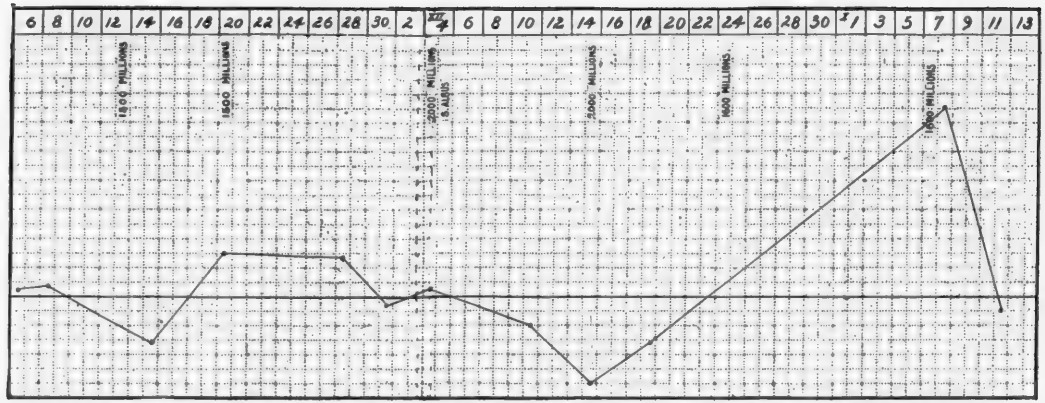

FIG. 7. Mr. A. Acne; recovery.

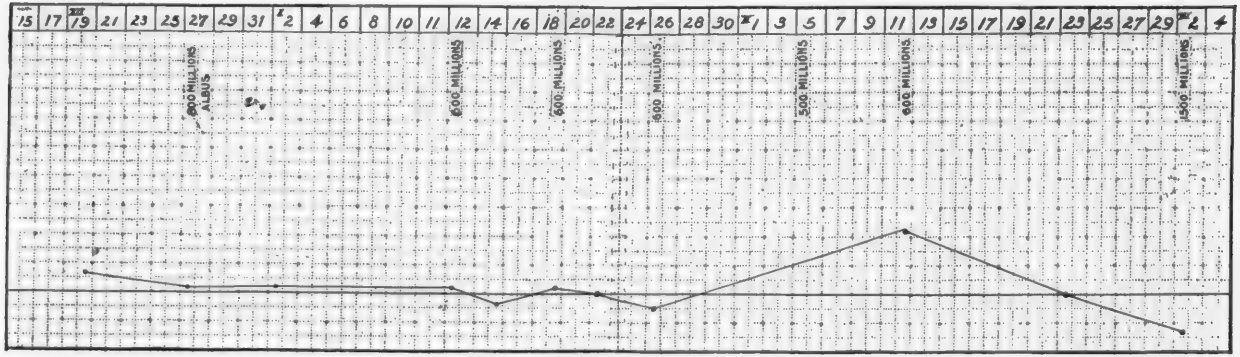

FIG. 8. Miss F. Acne; recovery.

tions in this direction have reference almost exclusively to tubercular and staphylococcus cases. I have already pointed out that many cases, of this kind, occur in which the index at the very outset of immunization is not materially lowered and that no very marked fluctuations may occur. In such cases successive inoculations of the corresponding vaccines brought about no material change in the height of the index, as compared with the normal. With small doses no negative phases were observed which could not readily be explained as being due to unavoidable errors of technique. Within a day or two there was usually (but not always) a rise in the index which then dropped again to near the normal line (Curve III, IV and V). A distinct and persistent rise was only observed in cases in which the nutrition of the patient was markedly below the normal at the start. As this improved (during the process of immunization) the index gradually rose to normal, without any marked fluctuations. In one instance (Curve VI) remarkable variations then occurred (the patient steadily improving), but it will be noted without a persistingly upward tendency.

In no instance was a continued high tide observed and after several months of active immunization the index was not found higher than normal (Curve VII and VIII). The patient from whom Curve IV was taken had been under vaccine treatment for nearly a year.

Animal experiments led to the same result. We may accordingly conclude that in the case of the tubercle bacillus and the staphylococcus a study of the opsonic content of the blood by Wright's method shows no evidence of the development of an opsonic immunity, which likewise argues against the specificity of the opsonins. The available literature having reference to this point is still too meager to furnish any data which could be interpreted as evidence either for or against this conclusion, but I note that Bulloch states that "in most cases the high tide is in the course of a few days succeeded by a fall again. . . . In spite of this the improvement may continue, a fact which leads me to assume that there are other factors at work which at the present time cannot be measured." This coincides with my own views and I would emphasize that no matter what the future of the opsonic "index"

may be there can be no doubt whatsoever that remarkably beneficial results can be reached by means of bacterial vaccines and that Wright deserves full credit for having generalized this principle of immunization in human therapeutics.

OPSONINS AND AGGRESSINS.

In order to ascertain whether the lowered opsonic content which may be observed in the various bacterial infections is referable to an insufficient production of opsonic material or to the simultaneous presence of inhibitory substances (aggressins, endotoxins), a series of experiments was undertaken in which the blood of infected individuals was added to normal blood and the phagocytic power of the mixed blood compared with the normal. My results are not yet numerous enough to warrant any definite conclusions, but they show that in some instances in which the patient's index was low, a distinct inhibitory effect occurs. That this is not the expression of dilution merely, with a serum of lowered bacterial content, is clear from the fact that the substitution of saline solution for the patient's blood does not cause a corresponding effect.

Example I.—The patient was a young girl with generalized septicæmia following an appendectomy. Her opsonic content on Nov. 25, measured by my percentage method, was 18; the normal control value was 72, and her percentage index accordingly .25. A mixture of equal volumes of the control serum and saline solution gave 32 per cent., corresponding to an index of .44, while a mixture of the patient's and the control serum gave only 16 (index .22). There was thus a drop of 50 per cent., which, I think, cannot be otherwise interpreted than as being due to the action of inhibitory substances in the patient's serum.

Such results, however, were exceptional and not constant even in one and the same individual. At times, indeed, the mixed sera gave higher values than the control, conveying the impression as though the normal serum had liberated a bound quotum of the patient's opsonins. If this idea of bound opsonins in the infected individual could be shown to hold good, it would explain the curious fact that notwithstanding the existence of such infections in which the process of phagocytosis is the only method of defense of which we have evidence, normal values are so frequently observed although active symptoms exist, from which we would expect to find the opsonins either high or low, but certainly not normal.

Example II.—On the sixteenth of February the percentage value of the patient referred to in Example I was 20, while the control was 72; her index hence was .27. The mixture of control and saline gave 40, *i. e.*, an index of .55, while that of the patient's and the control serum was 80, corresponding to an index of 1.11 and representing an increase of 100 per cent.

Example III.—In a woman following hysterorrhaphy a mixed staphylococcus and streptococcus septicæmia developed. Her percentage value for the staphylococcus was 76, while the control was exactly the same and her index hence 1.00. The mixture of the control and saline gave 60 (index .78), while that of the patient's serum and the control was 92 and the index hence 1.21, representing an increase of about 50 per cent.

Working with exudates I have repeatedly noted that their addition to normal serum causes a more marked drop in the phagocytic index than the corresponding blood serum. This is manifestly not due to a lower content in opsonic material *per se,* as transudates and cystic fluids likewise furnish lower values; but their admixture (when fresh) to normal serum does not bring about a corresponding decrease.

Example IV.—The percentage phagocytic value of a specimen of fresh hydrocele fluid was 12, while that of the normal blood serum was 72; the index thus was .16. The mixture of control serum and saline gave 52 (index .72) and that of the serum and hydrocele fluid 48, corresponding to an index of .66, thus showing no material effect.

Example V.—A specimen of fluid was obtained from an intraligamentary cyst; its phagocytic value was 16 per cent. and that of the control serum 76; the index hence .21. Equal parts of serum and saline gave 42 per cent. (index .55) and of saline and cystic fluid 48 per cent. (index .63).

Example VI.—Fluid from an ovarian cyst; phagocytic value 4 per cent. (index .10); equal parts of control serum and saline gave 32 per cent (index .80) and of saline and cystic fluid 28 per cent. (index .90).

There is thus no evidence of any inhibitory action whatever. But with exudates, as I have said, there is often a marked effect upon the phagocytic power which I hardly think can be interpreted otherwise than as being due to definite inhibitory substances.

Example VII.—Mrs. C., the patient referred to before (Example III). The staphylococcus value of the pleural exudate in this case was 48 (index .63), while with the streptococcus no phagocytosis whatever was observed. It is interesting to note that the exudate contained streptococci in large numbers, which were cultivated from the exudate and from the patient's blood, while the staphylococcus infection was apparently localized. The mixture of normal serum and saline for the staphylococcus gave 76 and for serum and exudate 80 (index 1.05), while with the streptococcus the values were 42 and 12 respectively, giving an index of .23, thus showing a decrease of over 70 per cent. (!), which, as I have said before, must be attributed to the action of inhibitory substances.

The following example shows a similar effect.

Example VIII.—The patient was an old gentleman, aet. about 70, with empyema. The phagocytic power of his blood serum was 88 per cent., corresponding to a control of 72, thus giving an index of 1.11; the value of the exudate was 12 and the index hence .16. Equal parts of normal serum and saline gave 60 per cent. (index .88) and of normal serum and exudate 12 per cent. (index .16) showing a drop of about 80 per cent.!

The patient died a few days later and the exudate which was drawn off after death again examined. Its direct phagocytic value was 4 (index .10); normal serum and saline gave 32 (index .80) and saline and exudate 8 (index .20), thus showing a decrease of 75 per cent.!

Example IX.—(Same patient as Example I.) Within forty-eight hours preceding death ascites developed and in the exudate chain cocci, diplococci and bacilli were found in immense numbers. Some of the material was freed from bacteria by prolonged centrifugation and then showed a staphyloccus value of 4 per cent. (index .16); normal serum and saline gave 16 per cent. and with serum and exudate no phagocytosis whatever occurred—a loss of 100 per cent.!!

Of the character of the inhibitory substances in the blood and in the exudates of infected individuals nothing is known. I have some evidence, which suggests that they are comparatively unstable, but am not prepared to discuss this phase of the subject at the present time. Whether or not they are of bacterial origin and of the nature of aggressins (viz., endotoxins), or whether they are products of autolysis referable to the cells of the infected individual future research will have to show, but their demonstration in the blood and exudates renders it possible, if not probable, that the low opsonic values which are so frequently seen in the various infections may be due in part at least to the simultaneous presence of antagonistic substances.

CONCLUSIONS.

1. The determination of Wright's index of the opsonic content of the blood and other fluids of the body is open to serious and in part unavoidable errors and should be abandoned in its present form.

2. Conclusions based upon the determination of the opsonic content of the blood, according to Wright's method, are accordingly not uniformly reliable.

3. The percentage index is a valuable check on Wright's bacillary index, but likewise does not furnish an adequate idea of the opsonic content of the blood, unless carried out with progressive dilutions to the point of opsonic extinction.

4. The opsonins of normal blood serum are not specific.

5. The specificity of the opsonins in "immune" sera has not been satisfactorily established, but appears probable.

6. An opsonic immunity, in the sense of a continued high opsonic content of the blood does not exist.

7. In the blood and exudates of infected individuals substances may be present which exercise an inhibitory effect upon phagocytosis.

OPSONINS OF INFLAMMATORY EXUDATES.[1]

By EUGENE L. OPIE.

(From the Rockefeller Institute for Medical Research, New York.)

There is much uncertainty concerning the relationship of so-called opsonins to other bodies concerned in the production of immunity and available data is insufficient to indicate with what accuracy the opsonic power of the serum measures resistance to bacteria either in normal or in immunized individuals. Wright has found the opsonic power of the serum with certain bacterial infections much below the normal and believes that a low opsonic index indicates susceptibility to the particular microörganism which has been employed in testing the opsonic activity of the blood serum. The low opsonic index observable with certain pyogenic and tuberculous infections is believed by Wright to antedate infection, being indeed its cause. There can be no doubt that the opsonic power of the blood is subject to considerable variation, but non-bacterial factors which depress it are but little known. Hektoen and Ruediger[2] have shown that a variety of inorganic salts inhibit the opsonic activity of the serum in the test-tube, but what effect similar substances have during life is not known.

Wright and Reid[3] further have shown that exudates produced by a given bacterium may exhibit partial or complete absence of opsonin for this microörganism. The fluid in the peritoneal cavity with tuberculous peritonitis may contain no opsonin for tubercle bacillus; it has, to use the expression of Wright and Reid, a low bacteriotropic tension.

The following experiment shows that an inflammatory exudate caused by one microörganism may contain no opsonin for this or for other microörganisms. A fatal quantity of Staphylococcus pyogenes aureus has been injected into the peritoneal cavity of the

[1] Received for Publication July 2, 1907.

[2] *Jour. of Infec. Diseases,* 1905, ii, 128.

[3] *Proc. of the Roy. Soc.,* 1906, lxxvii, 194.

guinea-pig and the power of the serum to opsonize Staphylococcus aureus and Bacillus typhi has been tested.

Experiment 1.—Into the peritoneal cavity of a guinea-pig were injected three cultures on agar-agar of Staphylococcus pyogenes aureus suspended in 3 c.c. of 0.85 per cent. sodium chloride. Death occurred in twenty-four hours; the peritoneal cavity contained 10 c.c. of turbid fluid from which clear serum was obtained by centrifugalization. The blood serum of a normal guinea-pig and 0.85 per cent. solution of sodium chloride were used for comparison. Since washed leucocytes of the guinea-pig are obtained with difficulty, corpuscles of the dog were employed. Bacteria used were suspended in 0.85 per cent. solution of sodium chloride. Each mixture contained equal volumes of its various ingredients. The following figures represent the average number of bacteria in a leucocyte after incubation at 38° C. during fifteen minutes.

With Staphylococcus pyogenes aureus:

Corpuscles + blood serum + staphylococci	16.0
Corpuscles + exuded serum + staphylococci	2.0
Corpuscles + salt solution + staphylococci	3.7

With Bacillus typhi:

Corpuscles + blood serum + B. typhi	8.44
Corpuscles + exuded serum + B. typhi	0.16
Corpuscles + salt solution + B. typhi	0.48

To determine if the serum of the exudate was capable of preventing phagocytosis in the presence of blood serum, phagocytosis in a mixture containing two volumes of normal blood serum was compared with phagocytosis in a similar mixture in which one volume of blood serum was replaced by a volume of exuded serum.

Corpuscles + blood serum + blood serum + staphylococci	9.2
Corpuscles + blood serum + exuded serum + staphylococci	11.5
Corpuscles + blood serum + blood serum + B. typhi	6.6
Corpuscles + blood serum + exuded serum + B. typhi	7.0

The experiment shows that opsonin for both Staphylococcus pyogenes aureus and for Bacillus typhi are absent in the exudate produced by intra-peritoneal injection of the first named micro-organism. The exudate thus produced (containing so-called aggressin) fails to inhibit phagocytosis when opsonin is supplied by addition of normal blood serum.

A second experiment with an exudate obtained by injecting the bacillus of swine plague into the pleural cavity of the rabbit gave a similar result. It is noteworthy that this organism, virulent for the

rabbit, failed to undergo phagocytosis in the presence of the blood serum of a normal rabbit, yet almost completely deprived the exudate of power to promote phagocytosis of Staphylococcus pyogenes aureus or of Bacillus dysenteriæ.

Experiment 2.—Into the right pleural cavity of a rabbit was injected one culture on agar-agar of Bacillus sui pestis; death occurred in about twenty hours. The exudate present in the pleural cavity was centrifugalized and tested with B. sui pestis, Staphylococcus pyogenes aureus and B. dysenteriæ. Blood serum of a normal rabbit and washed corpuscles of the dog were used. Each mixture contained four equal volumes as follows:

With Bacillus sui pestis:

Corpuscles + blood serum + salt solution + B. sui pestis.....	0
Corpuscles + exuded serum + salt solution + B. sui pestis.....	0
Corpuscles + blood serum + exuded serum + B. sui pestis.....	0.04

With Staphylococcus pyogenes aureus:

Corpuscles + blood serum + salt solution + staphylococci.....	10.4
Corpuscles + exuded serum + salt solution + staphylococci.....	2.2
Corpuscles + blood serum + exuded serum + staphylococci.....	22.7

With Bacillus dysenteriæ:

Corpuscles + blood serum + salt solution + B. dysenteriæ.....	2.4
Corpuscles + exuded serum + salt solution + B. dysenteriæ.....	0.0
Corpuscles + blood serum + exuded serum + B. dysenteriæ.....	0.3

With one exception (B. dysenteriæ, Experiment 2) more active phagocytosis has occurred in the presence of a mixture of both blood serum and exudate than in blood serum alone, and this increased activity has been observed even when a mixture containing one volume of blood serum and one volume of exuded serum has been compared with a mixture containing two volumes of blood serum. The following experiment confirms this observation.

Experiment 3.—In the peritoneal cavity of a guinea-pig, twenty-four hours after injection of four agar-agar cultures of B. typhi, were found 10 c.c. of turbid fluid. The following tests were made after centrifugalization, the blood serum of a normal guinea-pig and the washed leucocytes of a dog being used.

With Bacillus typhi:

Corpuscles + blood serum + salt solution + B. typhi.....	14.0
Corpuscles + blood serum + exuded serum + B.typhi.....	21.2

With Staphylococcus pyogenes aureus:

Corpuscles + blood serum + salt solution + staphylococci	16.0
Corpuscles + blood serum + exuded serum + staphylococci	26.1

The exuded serum was not tested with bacteria alone until it had stood for some time and the results are not therefore comparable with the foregoing.

Corpuscles + exuded serum + B. typhi	0.2
Corpuscles + exuded serum + staphylococci	3.74

Evidence that absence of opsonic activity may bear no relation to bacterial injection is furnished by examination of the sterile pus obtained by repeatedly injecting small quantities of turpentine into the pleural cavity of the dog.

Experiment 4.—Serum of pus was obtained by centrifugalization of pus removed from the pleural cavity of the dog after repeated injection of turpentine. Absence of bacteria in the exudate was demonstrated by the negative result of attempted cultivation and by examination of stained preparations. The opsonic content of the fluid part of the pus was tested by the usual method, washed corpuscles of the dog and a suspension of Staphylococcus pyogenes aureus being used. For comparison, activity of phagocytosis in the presence of blood serum of the dog and in physiological (0.85 per cent.) salt solution was determined.

Corpuscles + blood serum + staphylococci	5.5
Corpuscles + exuded serum + staphylococci	0.5
Corpuscles + salt solution + staphylococci	0.4

The following experiment confirms that just described and shows, moreover, that diminution of phagocytosis is due to loss of opsonic action and not to the inhibiting action of turpentine.

Experiment 5.—An abscess was produced by injecting one cubic centimeter of turpentine into the subcutaneous tissue of the dog. Serum was obtained by centrifugalization from the thick sterile pus present at the end of four days. The opsonic activity of this serum was compared with that of the blood.

Corpuscles + blood serum + staphylococci	1.9
Corpuscles + exuded serum + staphylococci	0.5
Corpuscles + salt solution + staphylococci	0.1
Corpuscles + blood serum + blood serum + staphylococci	2.7
Corpuscles + blood serum + exuded serum + staphylococci	2.8
Corpuscles + exuded serum + exuded serum + staphylococci	1.2

Phagocytosis with serum of the exudate is much less than with blood serum, but when serum of the exudate is mixed with blood serum, phagocytosis is undiminished.

With the serum of the sero-fibrinous pleurisy caused by turpentine injected into the pleural cavity phagocytosis is approximately equal to that which occurs in the presence of blood serum. The following experiment is cited to show that turpentine does not necessarily destroy opsonic activity.

Experiment 6.—Serum from a sero-fibrinous exudate withdrawn from the pleural cavity of the dog three days after injection of 2 c.c. of turpentine was freed from cells by centrifugalization.

Corpuscles + blood serum + staphylococci	10.7
Corpuscles + exuded serum + staphylococci	11.1

The experiments show that the serum of a purulent exudate obtained after the cells have been deposited by centrifugalization may contain no opsonin for staphylococci, even though it has been produced by a sterile irritant and contains no microörganisms. This fact is in agreement with the observation made upon exudates caused by bacteria injected into the guinea-pig and rabbit, for here injection of one microörganism caused loss of opsonin for the other microörganisms which were tested. Nevertheless the experiments do not demonstrate absence of specificity on the part of opsonins, and in Experiment 3 a certain degree of specificity is perhaps suggested, injection of typhoid bacillus causing almost complete disappearance of opsonin for this microörganism, but only partial disappearance of opsonin for staphylococcus.

Absence of opsonin in the exudate produced by an inflammatory irritant of great activity has no necessary relation to the nature of the irritant, for in a purulent exudate caused by a sterile irritant there may be disappearance of opsonin for staphylococci. It is not improbable that the complement-like opsonin of normal sera, which, as Muir and Martin[4] have shown, is absorbed by a great many substances exhibiting combining affinities for complement, has been absorbed in the experiments just described not only by microörganisms but by cells contained in immense number in the purulent exudate, for it is well known that various tissue extracts can absorb complement.

The observations which have been described probably explain in part the presence of the innumerable extracellular bacteria which may be found in the serum of many purulent exudates. With a microörganism such as the meningococcus, streptococcus and staphylococcus, which readily undergoes phagocytosis in the body or *in vitro,* it is possible that exhaustion of opsonic content of the fluids, rather than impaired phagocytic power of the cells, may explain the occurrence of uningested microörganisms in considerable number in the exudate produced by these bacteria.

[4] *British Med. Jour.,* 1906, ii, 1783; *Proc. Roy Soc.,* 1907, B. lxxix, 187.

EXPERIMENTAL LIVER NECROSIS; I. THE HEXON BASES.[1]

By HOLMES C. JACKSON, Ph. D.,
Adjunct Professor of Physiological Chemistry,
AND
RICHARD M. PEARCE, M.D.,
Professor of Pathology and Bacteriology,
Albany Medical College.

(*From the Bender Laboratory, Albany, N. Y.*).

This communication is the first of a series of five, which presents the results of a somewhat comprehensive investigation of the chemical changes associated with experimental liver necrosis and which includes, in addition to that here presented, studies of the nitrogenous metabolism, nuclein metabolism, the activity of the intracellular hepatic enzymes and the changes in the fats and lipoids of the liver.

The advantage of undertaking such a comprehensive study was suggested by the investigations[2] which one of us had previously made of the necrosis caused in the liver of the dog by the intravenous or intraperitoneal injection of hæmagglutinative and hæmolytic immune sera. The lesions so produced are frequently focal and resemble in a general way those of eclampsia while the diffuse lesions, with the associated repair, are more or less similar to certain stages of acute yellow atrophy. It seemed plausible therefore that a study of the chemistry of such lesions, readily produced experimentally, might throw light not only on certain functions of the liver but might offer new knowledge of value in explaining

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Read by title before the Society of Experimental Biology and Medicine, New York, March 20, 1907. Received for publication July 2, 1907.

[2] Pearce, R. M., The Experimental Production of Liver Necroses by the Intravenous Injection of Hæmagglutinins, *Jour. of Med. Research,* 1904, xii, 329; 1906, xiv, 541. Experimental Cirrhosis of the Liver, *Jour. of Exper. Med.,* 1906, viii, 64.

some of the problems of eclampsia, acute yellow atrophy and similar lesions in man.

The study of the hexon bases was undertaken in the hope of determining the character of the processes underlying the production of *intra vitam* autolysis. We have therefore attempted to determine the relative enzymotic power of the normal and the necrotic liver in regard to the synthesis or decomposition of the hexon bases. The importance of these bodies, as products of autolysis, has been emphasized by Wakeman[3] in his study of the livers of dogs poisoned with phosphorus or repeatedly anæsthetized with chloroform. In addition to the study of fresh tissues, we have investigated also the changes occurring in hexon nitrogen during antiseptic autolysis of liver substance.

The experimental lesions under consideration may be described, without going into detail, as hyaline necroses with little or no leucocytic reaction, the position and extent of which vary according to the amount of serum administered and the resistance of the animal. Small doses cause focal lesions more or less isolated and irregularly distributed; large doses produce a diffuse necrosis which spares only the tissue about the larger portal spaces. The lesions are found chiefly near the surface of the liver, but may occur in the deeper portions. In animals dying within a few hours after injection and before the appearance of necrosis an intense congestion of the entire portal system exists with, in the liver, innumerable thrombi composed of fused red cells.

In addition to such material from the dog, the livers of horses presenting degenerative and necrotic lesions, occurring in the course of immunization with various bacterial products, have also been utilized. These lesions, which have been described fully elsewhere by Pease and Pearce[4] and more recently by Lewis,[5] consist of widespread necrosis associated not infrequently with an extensive deposition of amyloid and usually accompanied by hæmorrhages.

[3] Wakeman, A. J., On the Hexon Bases of Liver Tissue under Normal and Certain Pathological Conditions, *Jour. of Exper. Med.*, 1905, vii, 292.

[4] Pease, H. D., and Pearce, R. M., Liver Necrosis and Venous Thrombosis in Horses Actively Immunized with Diphtheria and Tetanus Toxins and with Streptococci and their Products, *Journ. of Infect. Diseases*, 1906, iii, 619.

[5] Lewis, P. A., Hæmorrhagic Hepatitis in Antitoxin Horses, *Jour. of Med. Research*, 1906, xv, 449.

A series of fifteen livers, eleven from the dog and four from the horse, has been examined. Of the former, five were normal, four showed focal necroses and two diffuse necrosis; of the latter two were normal and two were examples of necrosis associated with extensive amyloid transformation.

Methods.—As the necrotic lesion in the dog's liver reaches its greatest extent in twenty-four to forty-eight hours after injection, all livers used in the study of the hexon bases were taken from animals killed at the end of the latter period. In the case of the horses the livers were removed within one hour after death.

After cutting away the larger vessels and the coarser tissue about the hilum the organ was subdivided and passed through the hashing machine. Small portions were weighed and set aside for drying and upon these total nitrogen estimations were made later. At the same time two portions of 100 grams each of moist liver were placed in flasks. To one, 600 cubic centimeters of water and 300 grams of sulphuric acid were added and hydrolysis carried on for fourteen hours in a paraffin bath. The other sample was suspended in 600 cubic centimeters of water and with it were thoroughly mixed about fifty cubic centimeters of toluol. This flask plugged with cotton was allowed to stand at room temperature for periods of one or two months. At the end of this time it was examined bacteriologically, and if found free of bacteria, 300 grams of sulphuric acid were added and the material hydrolyzed in the same manner as the control. Subsequent treatment was carried out according to the method outlined by Wakeman except that the process was continued only to the point of making nitrogen determinations of fractions "A" (arginin and histidin) and "B" (lysin) after removal of the barium and silver by means of sulphuric acid and hydrogen sulphide. For our purpose no advantage was to be gained by separating arginin and histidin.

Dry Solid Content.—An examination of the figures in the table indicates that the dry solid content of the dog livers with scattered focal necroses did not vary from the normal average of 24 per cent. In the case of the diffuse lesions, where the autolysis of the protoplasm was obviously more marked, the dry solids were reduced to an average of 20.8 per cent. The figures referring to normal livers

TABLE—*Hexon Bases.*

Nature of Experiment.		Determination on Fresh Tissue.						On Autolysed Tissue.			
		Per Cent.			* Per Cent. Nitrogen as				* Per Cent. Nitrogen as		
Dog.	Condition of Liver.	Dry Substance.	Nitrogen in Dry Substance.	N_2 ppt. by Phospho-tung. Acid.	Arginin and Histidin.	Lysin.	Purin Bases.	Per Cent. of N_2 ppt. by Phospho-tung. Acid.	Arginin and Histidin.	Lysin.	Purin. Bases.
3	Normal	24.8	12.1	13.2	4.3 32.5	3.6 27.1	5.3 40.4	Not bacteria-free			
4	Normal	18.4	10.9	15.0	6.0 40.0	4.9 32.7	4.1 27.3	19.8	8.4 42.4	9.8 49.5	1.6 8.1
13	Normal	26.8	11.8	Used only for autolysis				18.3	8.0 43.7	7.3 39.9	3.0 16.4
14	Normal	24.5	9.2	Used only for autolysis				17.6	7.5 42.6	7.6 48.2	2.5 14.2
17	Normal	25.0	11.2	19.6	4.3 36.2	7.4 37.7	5.1 26.1	20.4	8.0 39.2	7.9 38.7	4.5 22.1
Av.		23.9	11.3	15.9	6.0 36.2	5.3 32.5	4.8 31.3	19.0	8.0 42.0	8.2 42.8	2.9 15.2
5	Few focal necroses	24.4	21.4	12.2	5.6 45.8	4.2 34.4	2.4 19.8	9.0	4.1 45.5	2.7 30.0	2.0 24.5
7	Few focal necroses	16.6	19.1	14.5	7.3 50.2	5.1 35.1	2.1 14.7	10.2	4.9 48.0	3.5 34.3	1.9 17.7
9	Numerous focal necroses	26.9	22.8	10.2	4.8 47.2	3.2 31.5	2.1 21.3	6.7	2.3 34.3	2.6 38.8	1.8 26.9
10	Numerous focal necroses	25.6	23.7	8.5	3.6 42.3	2.5 29.4	2.4 28.3	6.8	2.7 39.8	1.9 27.9	2.1 32.3
Av.		23.4	21.8	11.4	5.3 46.4	3.8 32.6	2.2 21.0	8.2	3.5 41.9	2.7 32.8	2.0 25.3
29	Extensive diffuse necrosis	21.9	12.7	30.0	15.6 52.0	11.6 38.6	2.8 9.4	23.9	11.5 48.1	10.2 42.7	2.2 9.2
60	Extensive diffuse necrosis	19.8	13.2	25.6	12.7 49.6	11.3 44.1	1.6 5.3	20.7	10.1 48.8	9.5 45.9	1.1 5.3
Av.		20.8	12.95	27.8	14.2 50.8	11.5 41.4	2.2 7.4	22.3	10.8 48.5	9.9 44.3	1.7 7.3

TABLE—*Hexon Bases (Continued)*.

Nature of Experiment.		Determination on Fresh Tissue.					
		Per Cent.			* Per Cent. Nitrogen as		
Horse.	Condition of Liver.	Dry Substance.	Nitrogen in Dry Substance.	N_2 ppt. by Phosphotungstic. Acid.	Arginin and Histidin.	Lysin.	Purin Bases.
60	Normal	23.3	12.0	8.1	3.0 / 37.0	4.6 / 56.7	0.5 / 6.3
65	Normal	21.8	11.2	8.5	3.9 / 45.9	3.1 / 36.5	1.0 / 17.6
Av.		22.6	11.6	8.3	3.5 / 41.5	3.9 / 46.6	0.75 / 12.0
42	Necrosis and amyloid	20.0	21.7	13.5	2.9 / 21.4	8.2 / 60.7	2.4 / 17.9
69	Necrosis and amyloid	21.4	13.7	13.3	1.2 / 9.0	10.2 / 76.7	1.9 / 14.3
Av.		20.7	17.7	13.4	2.1 / 15.2	9.2 / 68.7	2.2 / 16.1

* Figures in upper left hand corner are calculated on the total nitrogen; those in the lower right hand corner on the total hexon nitrogen.

agree somewhat closely with those of Wakeman, who, however, noticed a slight decrease in the percentage of dry substance in the livers of dogs poisoned by phosphorus. This is somewhat surprising since he describes these livers as markedly fatty. If the protoplasm of which seventy-five per cent. is water is replaced by fat, which contains none, the dry solids should increase instead of decrease. If Wakeman's figures are correct they show a marked increase in the water content of the organs during phosphorus poisoning. That such an increase may occur did not appear in our investigation[6] of fatty changes in the liver.

Total Nitrogen Content.—Several interesting facts developed from the analysis of the nitrogen of the dry solids. The average of the five determinations for normal tissue was 11.3 per cent. in agreement with Wakeman; while that of the livers with lesions of a scattered focal character was 21.8 per cent.

This, of course, indicates a deposition or heaping-up of nitrogenous material in the hepatic cell. That such a process may take place even under physiological conditions is evident from the results of experiments carried out by Seitz.[7] This investigator found that by feeding hens and geese excessive amounts of meat a true deposition of nitrogenous substances occurred in the cells of the liver. This increase amounted in some instances to 300 per cent.

In our experiments the quantity of nitrogen in the hepatic cell was almost doubled in the organs with scattered necrotic lesions. This condition allows of an explanation similar to that offered for the infiltration of fat in tissues during phosphorus or phloridzin poisoning. In such lesions the cells have lost in part their power to oxidize properly the sugar or other materials placed at their disposal by the circulating blood and hence the starving cell, in its endeavor to spare its own protoplasm from destruction, stores up fat for purposes of oxidization.

That the cells at the margin of the necroses under consideration do accumulate fat we have observed in our study of the histological changes occurring in these livers.[6] It seems distinctly possible that

[6] See fifth paper of this series, "The Fats and Lipoids" in this number of the *Journal.*

[7] Seitz, W., Die Leber als Vorrathskammer für Eiweissstoffe, *Arch. f. ges. Physiol.*, 1906, cxi, 309.

during the initial stage of congestion and thrombosis and in the early stages of necrosis, the imperfectly nourished and slightly injured cells may heap up nitrogenous material also. When, however, the lesion is more extensive, the storing up of nitrogen is not so evident as shown by the fact that the nitrogen of the dry solids remains more nearly normal. This is to be explained by assuming that the nitrogen stored up in the persisting liver cells is sufficient to more than balance the loss by autolysis in the necrotic areas.

Wakeman's figures for the nitrogen content of livers after phosphorus poisoning show a diminution equivalent to 35.6 per cent. and a corresponding decrease in the hexon base nitrogen. This indicates, according to his view, that that part of the proteid molecule involving the hexon bases has not undergone a relatively greater decomposition than the other nitrogenous substances. It would seem to us that the low nitrogen content of the phosphorus livers is wholly, or in greatest part, due to the large amount of fat present.

The Hexon Bases.—Wakeman's results indicate that in the dog the average nitrogen content of the bases in the normal liver tissue is 17.04 per cent. of the total nitrogen, while in the liver of dogs poisoned with phosphorus it is only 10.72, a falling off of 37.1 per cent. The livers of dogs receiving chloroform showed 13.6 per cent., a decrease of 20 per cent. In these figures Wakeman sees evidence of increased autolysis in hepatic cells affected by phosphorus or chloroform. Although he mentions definite necrosis in but one of his livers, the cell destruction in phosphorus poisoning, gradual as it is, is such that our results ought to fall, as they do, somewhat into line with his.

Thus as an average of three normal livers we find the figures concerning the total content of hexon bases, based on nitrogen content attributable to them, to be 15.9 per cent. of the total nitrogen. In the case of the scattered focal necrosis, the percentage is slightly decreased to 11.4 as an average of four determinations, but the absolute amount is increased. That is gram for gram of dry substance there occurs an increase in the absolute amount of hexon bases which however appears as a decrease in percentage on account of the high nitrogen content of the dry substance.

This absolute increase in hexon content of dry substance is greater and more clearly accentuated in the livers of those dogs in which the necrosis is more diffuse. Here the nitrogen of the dry substance is almost the same as that of the normal, 12.95 per cent. as average of two determinations; but the hexon base nitrogen content rises to 27.8 per cent. of the nitrogen of the dry substance.

This observation is extremely interesting in that it points most strongly to the preponderance of the autolytic process over the synthetic in the more widespread forms of necrosis with early repair. The figures show a definite increase in the hexon base content of the necrotic cell, although the accumulation of nitrogen in this lesion, 12.95 per cent., could not occur to such a marked extent as it did in the focal lesion, 21.8 per cent., because of the lessened number of persisting living cells capable of storing up nitrogen. A rearrangement of nitrogen, the result of autolysis in the larger areas of necrosis, therefore took place as shown by the hexon nitrogen content of 27.8 per cent., as compared with that of 11.44 per cent. in the focal lesion and 15.9 per cent. in the normal. This great increase of hexon bases may be due in part also to disturbances of the circulation accompanying the necrosis which prevent the diffusion and removal of the bases from the liver. In this connection attention may be called to Jacoby's[8] observation that leucin and tyrosin are not found in the liver of phosphorus poisoning when no disturbance of the hepatic circulation exists.

Relation of Precipitate "A" (Arginin and Histidin) and Precipitate "B" (Lysin) to the Total Hexons.—Wakeman, from a consideration of his results on these fractions, concludes that in the autolysis which occurs in the cell in phosphorus poisoning the arginin suffers a greater destruction than do the other bases, probably through the action of arginase, which splits arginin into ornithin and urea.[9] His tables show that of the 17.0 per cent. of the nitrogen of the total bases in the normal tissues, 11.8 per cent. is to be attributed to arginin and histidin and 5.2 to lysin. In phosphorus poisoning, on the other hand, the nitrogen of the bases amounts to

[8] Jacoby, M., Ueber die Beziehungen der Leber und Blutveränderungen bei Phosphorvergiftung zur Autolyse, *Zeit. f. physiol. Chem.*, 1900, xxx, 174.

[9] Kossel, A., and Dakin, H. D., Ueber die Arginase, *Zeit. f. physiol. Chem.*, 1904, xli, 321.

10.7 per cent., of which only 6.8 per cent. belongs to the arginin and histidin and 3.8 per cent. to the lysin. This indicates a decrease during autolysis of 42.3 per cent. for the arginin and histidin, but only 26.8 per cent. for the lysin.

If, however, one considers these figures from the standpoint of the relationship which the precipitates "A" and "B" bear to the total hexon bases of the normal and phosphorus dogs, an entirely different view is obtained. In the normal tissue precipitate "A" (arginin and histidin) forms 69.6 per cent. and precipitate "B" (lysin) 30.4 per cent. of the total bases; whereas in the phosphorus livers the former is 63.9 per cent. and the latter 36.1 per cent. Hence the decrease in the fraction "A" is only 5.7 per cent. and this is offset by the corresponding increase in fraction "B." This diminution in the arginin and histidin content of the hexon base fraction of the livers of phosphorus-poisoned animals compared with the normal is so slight that it hardly seems warrantable to attribute it to the action of arginase.

Our figures for the total hexon bases (15.9 per cent.) in the normal agree well with those found by Wakeman, while those for the focal necroses have suffered a percentage decrease which is somewhat comparable to that noticed by him in phosphorus poisoning. Wakeman's percentage decrease, however, was also an absolute one while ours in reality was an absolute increase, as has been explained above. The proportion which precipitates "A" and "B" bear to the total is markedly different, however, from that which he notes. Our average in the normal tissues for the arginin and histidin fraction is 36.2 per cent. as against 69.4; the lysin fraction 32.5 per cent. more nearly agrees with his 30.3 per cent. If we exclude the purin bases our results become more comparable and agree better in percentage.

We have also found a much greater percentage of the total nitrogen due to purin bases. Wakeman's figures show an average of 0.0273 per cent. for normal and pathological, while ours showed 4.8 per cent. for the normal tissue against 2.2 per cent. for the focal necrotic lesions and the same for the diffuse necrosis.

As to variations which the individual bases undergo in their relation to the total bases, our results point to an increase from the

normal of 36.2 per cent. to 46.4 during the focal necrosis and to 50.8 per cent. during diffuse necrosis for arginin and histidin. The lysin fraction shows no change in the focal necroses as compared with the normal, but in the diffuse necrosis it increased 24.3 over the normal in agreement with Wakeman.

In the autolysis *in vitro*, which is discussed in the next paragraph, the normal fraction "A" represented 42.0 per cent. of the whole bases and fraction "B" 42.8 per cent.—differences from the normal which are well within the limit of error. Hence the absolute increase from 6.0 and 5.3 in the unautolyzed to 8.0 and 8.2 per cent. of the total nitrogen in the tissue after autolysis was in exact relation to the increase in total hexon nitrogen. The same is true for the autolyzed tissue with necrosis of all types.

This would emphasize more markedly the point made above that small evidence can be adduced to show that an enzyme, arginase, is acting on the arginin, decreasing its amount during autolysis. Such action is not shown by our figures and moreover in our investigation of intracellular hepatic enzymes[10] we could not obtain an active arginase from the necrotic dog's liver, though it was found in the normal.

Hexons Resulting from Autolysis in Vitro.—It seemed worth while in view of the investigation, carried on synchronously, of intracellular hepatic enzymes[10] to determine the relation of the hexon bases to autolysis of the liver *in vitro*. For such observations the figures given above for autolysis during life serve as controls. Autolysis was allowed to proceed for varying lengths of time in the endeavor to determine whether the different organs showed varied degrees of autolysis. The periods selected, one and two months, were inadequate to bring out this point, since the autolysis was completed or the reaction reached its equilibrium before one month. This was unfortunate as we thereby disregarded the important element, that of time, in this connection. The time element, however, is fully considered elsewhere[10] from another point of view.

A glance at the figures in the table shows that after autolysis of the normal organs the percentage of total nitrogen as hexon

[10] See second paper of this series, "Enzymes," in this number of the Journal.

nitrogen was 19.0 per cent. as an average of four determinations. This increase of 18.8 per cent. over the normal hexon content is not marked, and if one examines the figures referring to the two dogs (4 and 17) upon which alone we have absolute controls, it will be seen that this increase is variable.

We are not inclined to attempt to explain this result in detail in this place, since the data which we will present in our study of the enzymes bear more decisively upon this matter. Suffice it to say that these figures, taken in connection with those of the diffusely necrotic organs where an increase also was evident, indicate that in the autolysis a transformation or rearrangement occurs by which nitrogenous atomic complexes, not normally yielding hexon bases, become altered into hexon bases or their combinations.

In all degrees of necrosis, the autolyzed material contained a smaller amount of hexon bases than the unautolyzed. In the liver with scattered focal necroses the decrease of the hexon nitrogen in per cent. of the total nitrogen amounted to 28.0 per cent.; in the diffuse necrosis 19.8 per cent. This would seem to imply that in the living tissue the hexon splitting enzyme is to some degree inhibited, probably through the action of the blood serum.

Hexon Bases in the Liver of the Horse.—The results obtained with the normal livers of horses agree in regard to the dry solids (22.6 per cent.) and the nitrogen of the dry tissue (11.6 per cent.) with those obtained for the dog. The nitrogen precipitable with phosphotungstic acid, however, is surprisingly low, amounting to only 8.3 per cent. It would seem inadvisable to attempt an explanation of this difference, since these animals were not absolutely normal, in that they had been utilized for the purpose of preparing antitoxin and had died during such treatment, though no lesions were found in the liver. The injection of bacterial products may set up processes in the cell which tend to reduce its hexon content without changes evident histologically. The percentage which fractions "A" and "B" of such livers bears to the total hexon content is not far removed from that found for each in the case of the normal dog liver.

The two lesions which served as examples of necrosis presented the complicating feature of amyloid. This fact renders the series

not exactly comparable to the previous one. The increase in nitrogen of the dry substance over that of the normal is present here also, and the total hexon base nitrogen has increased to 13.4 per cent. This latter result may be caused by the heaping up of the bases as products of autolysis in the large necrotic areas. More probably, however, it is to be attributed to the amyloid degeneration, in which the normal cellular proteids with relatively small percentage (10–30 per cent.) of diamino-nitrogen are replaced by amyloid, the proteid constituent of which, in the liver and spleen as shown by Neuberg,[11] contains twice as much (50–60 per cent.) diamino-acid nitrogen. This rearrangement in the hexon content of the proteids of the organ has also resulted in a marked change in the relation which the arginin and histidin as well as the lysin bears to the total amount of hexon base precipitate. "A" forms only 15.2 per cent. and precipitate "B" 68.7 per cent. of the whole diamino-acid nitrogen.

SUMMARY.

1. The liver of the dog in which necrosis has been produced by injection of hæmatoxic immune sera is characterized in the less marked forms by a storing up of nitrogen in the persisting living cells, while in the diffuse forms the total nitrogen content is but slightly above the normal. This last is to be explained by the great diminution in persisting liver substance which limits the power of nitrogen accumulation.

2. In all forms of necrosis there occurs an absolute increase of nitrogen precipitable by phosphotungstic acid (hexon bases) but the percentage increase, in relation to total nitrogen, diminishes in those forms (focal) in which the products of autolysis may be readily carried off by the blood stream and greatly increases in the diffuse form with large areas in which the circulation is seriously impaired.

3. Although the absolute amount of nitrogen representing arginin and histidin varies, a relative increase is evident when this fraction is compared with the total diamino-nitrogen. This increase corresponds to the degree of necrosis and attendant circu-

[11] Neuberg, Ueber Amyloid, *Verhand. d. Deut. path. Gesellsch.*, 1904, vii, 19.

latory disturbance and indicates that in necrosis as opposed to degeneration (Wakeman) arginin is not split up by arginase. The lysin also bears a definite relation to the total hexon nitrogen.

4. The diamino-nitrogen of the normal liver after autolysis *in vitro* shows a slight variable increase over that of the unautolyzed, while the necrotic livers showed a decided decrease.

5. The diamino-acid nitrogen of normal horse liver is only about one half of that of the dog; the relative proportion of the bases is about the same. In necrotic livers with amyloid the diamino-nitrogen is markedly increased which is in accord with Neuberg's observations on the high hexon base content of amyloid.

Conclusions.—Upon the whole then the chemical processes occurring in the hepatic cell undergoing rapid or immediate necrosis and those accompanying a slow "degeneration," as for example in phosphorus poisoning, must be different and distinct as would be expected from the histological findings. In necrosis we find the cell in a complete state of disorganization and decomposition and hence autolysis begins immediately, but in the changes occurring in the cell in the so-called degenerations, as phosphorus poisoning, the nucleus remains intact, thereby insuring to a certain extent the life or at least partial function of the cell. That under the latter circumstance a disturbed condition does exist is evidenced by the heaping up of fat in the cell, and although the results of the various investigations upon the altered processes in the liver of animals poisoned with phosphorus tend to show that this change is an autolysis, in which certain amino-acids appear as the result of the splitting of the proteid molecule, it is not of the same type as that appearing in the necrotic cell. This is shown by a comparison of Wakeman's findings, which indicate definitely a diminution of hexons in the liver, with ours which show a great increase.

Examinations of the human liver, by a direct method without hydrolysis, but few in number, it is true, tend to the same conclusion. Taylor,[12] for example, found arginin in a liver with wide-

[12] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskörper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

spread necrosis, the result apparently of chloroform poisoning; from seven other livers, representing various lesions (dysenteric abscess, fatty degeneration, pyæmia and acute yellow atrophy), he was unable to isolate this substance. Soetbeer[13] also was unable to find hexon bases in a peculiar type of cirrhosis with acute degeneration.

This difference in the hexon content of the liver of "degeneration" and that of necrosis is so striking that it would appear to be due to a difference in the nature or rapidity of the cell destruction, though it may to some extent be explained by the disturbances of circulation which occur in the necrosis and which, presumably, are absent in degeneration.

Our observations show also that care must be exercised in drawing conclusions from results obtained by autolysis *in vitro*. It seems distinctly doubtful whether the autolysis of the cell which occurs under such circumstances has any relation to autolytic changes during life.

[13] Soetbeer, F., Ueber einen Fall von akuten Degeneration des Leberparenchyms, *Arch. f. exper. Path. u. Pharm.*, 1903, l, 294.

EXPERIMENTAL LIVER NECROSIS; II. ENZYMES.[1]

By RICHARD M. PEARCE, M.D.,
Professor of Pathology and Bacteriology.

AND

HOLMES C. JACKSON, Ph.D.,
Adjunct Professor of Physiological Chemistry.
Albany Medical College.

(*From the Bender Laboratory, Albany, N. Y.*).

The experiments about to be described represent an attempt to determine the relation of the intracellular hepatic enzymes to chemical changes occurring in liver necrosis. Our results are based on a comparison of the variations in the enzymotic equilibrium of the normal hepatic cells with those occurring in necrosis of varying grades of severity. At present the chief and most promising method of detecting such variations consists in determining by means of post-mortem autolysis the condition under which the cell is existing at the time of the death of the animal, and the rapidity, nature and extent of the changes which occur after the commencement of the autolysis. We are well aware that the interpretation of the results of post-mortem autolysis in relation to cellular activity during life is open to objection and may not have the importance usually ascribed to it.

Our investigation of the enzymotic activity of the liver tissue under normal circumstances and in varying degrees of necrosis may naturally be subdivided as follows:

1. A determination in a quantitative way of the degree of autolysis which the tissue undergoes after death.

2. A study of the individual enzymes with reference to the part which they play in the general course of autolysis.

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3. A determination of the products formed as the result of such autolysis. These include the diamino-acids, which have been considered in the preceding paper,[2] where they more properly belong, and the monamino-acids to which, as represented by leucin and tyrosin, we have given considerable attention. It was our intention to determine, by perfusion of livers in various stages of necrosis, the changes in the composition of the blood which might occur, but owing to the great amount of labor entailed in the present studies this has been unavoidably postponed.

Comparative Estimation of Products of Autolysis.—In the study of the changes which the nitrogenous material undergoes during autolysis *in vitro* an attempt was made to carry out a partition analysis of the non-coagulable nitrogen. This method has already been employed with good results by v. Drjewezki[3] to determine the effect of alkalies of varying strengths upon autolysis. His results, as well as those of Wiener,[4] point to the sensitiveness of the autolytic enzymes to changes in reaction, especially those due to alkalies, and these investigators conclude that the alkalies of the serum are responsible for the well-known inhibitory effect of the serum upon autolysis. Baer[5] and his associate, Loeb,[6] admit the inhibitory effect of the serum but are inclined to attribute it to the action of the serum globulin.

These facts, as well as those brought out by Lang[7] concerning the inhibitory effect of large quantities of toluol upon autolytic processes, although other factors may have influenced the results of the latter, all tend to emphasize the fact that in performing experiments of this character too much attention cannot be given

[2] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[3] v. Drjewezki, A., Ueber den Einfluss der alkalischen Reaktion auf die autolytischen Vorgänge in der Leber, *Biochem. Zeit.*, 1906, i, 229.

[4] Wiener, H., Ueber den Einfluss der Reaktion auf autolytische Vorgänge, *Zent. f. Physiol.*, 1905, xix, 349.

[5] Baer, J., Ueber die Wirkung des Serums auf die intracellularen Ferments, *Arch. f. exper. Path. u. Pharm.*, 1906, lvi, 68.

[6] Baer, J. and Loeb, A., Ueber die Bedingungen der autolytischen Eiweissspaltung in der Leber, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 1.

[7] Lang, S., Ueber desamidierung im Tierkörper, *Beit. z. chem. Physiol. u. Path.*, 1904, v, 321.

to the attainment of absolutely comparable conditions in all the various experiments. With these points in mind we have endeavored to control our work in every way, as is shown in the following detail of the experiments.

Quantities of the fresh tissue of known weight were ground to such a state of subdivision that when mixed with water or neutral Ringer's solution the mixture could be readily drawn up in a pipette. This mixture, usually consisting of two hundred grams of liver, was made up to 1,200 cubic centimeters and placed in a sterile flask and the mixture covered with a layer of toluol. This latter substance was well shaken in, after which were pipetted off, as controls, two samples of two hundred cubic centimeters each. Both were thoroughly sterilized in the autoclave in order to stop autolysis; one was examined immediately, as an initial control, the other was placed with the original mixture in the thermostat (37.5° C.) and examined at the conclusion of the experiment as a final control. The material in the thermostat was shaken from time to time and at intervals of one, three, five and eight days samples of the main mixture were removed for analysis by the same method as the controls. The analysis of the samples, after they were shown to be bacteria free,[8] took place in the following manner:

The mixture was pipetted into a beaker and sufficient water was added to allow of easy coagulation of the proteid material present. Acetic acid was added to slightly acid reaction after the boiling point was reached. The coagulated proteid was removed by filtration and repeatedly and thoroughly washed with boiling water. The volume of the filtrate and washings was made up to eight hundred cubic centimeters. Of this, twenty-five cubic centimeters served for the determination of the total nitrogen by the Kjeldahl-Gunning method, one hundred for the estimation of ammonia according to the method of Shaffer as applied to the urine, one hundred for the uric acid determination, using the Hopkins-Folin method, and fifty to determine the amount of nitrogen not precipitable by phosphotungstic acid in sulphuric acid solution, the so-called

[8] For this purpose it was deemed sufficient merely to examine stained films though when the final sample of each mixture was taken cultures were made. In this series representing nine livers no contamination occurred.

monamino-nitrogen. An attempt was also made to determine the nitrogen precipitable (proteoses) by zinc sulphate but our results are so incomplete that little can be gained from their discussion. In all cases duplicate determinations were made and the figures given represent their average. Dogs were employed in all experiments.

In Table I are presented the results of the nine experiments which differed in their conditions for the purposes of control, as follows:

Two normal livers (52 and 54) with their usual blood content, the diluting fluid of one being distilled water and of the other neutral Ringer's solution. This solution was prepared in the ordinary way with the exception that the sodium bicarbonate was not added in order to avoid an alkaline medium.

Two normal livers (53 and 58) washed *in situ*, the one with water and the other with neutral Ringer's solution.

One necrotic liver four hours after injection (57). An attempt was made to wash this liver with water but on account of the extensive thrombosis it was only partly successful. It is therefore referred to as "half-washed."

Two necrotic livers forty-eight hours after injection (48 and 56); one unwashed diluted with water; the other washed and diluted with neutral Ringer's solution.

Two livers (43 and 49) five days after injection, both showing necrotic lesions with early repair; one washed and diluted with Ringer's, the other unwashed but diluted with water.

In each instance in which the livers were washed the procedure was begun under ether while the animal was alive. With the exception noted the livers were completely blanched save for slightly tinged areas about the more diffuse foci of necrosis.

The results in Table I, in terms of nitrogen, are expressed in percentages of the total nitrogen of the dry tissue and of the total non-coagulable nitrogen. A critical consideration of the figures presented allows of the following statements:

Non-coagulable Nitrogen.—The inhibiting effect of the blood serum upon the extent of the autolysis of both normal and necrotic tissue is decisively shown. The percentage of non-coagulable nitrogen in the case of the unwashed normal organs increased from 10.7 and 9.7 to 19.9 and 29.1 per cent. respectively, an increase of 100 and 200 per cent., on the eighth day; while in the washed normal livers the average increase at the eighth day amounted to 450 per cent. The increase in the five day necrotic unwashed liver was 127 per cent.; that of the washed tissue 349 per cent. The forty-

TABLE I.

Autolysis; nitrogen partition.

Normal.				4 Hours.	48 Hours. Necrosis.		5 Days. Necrosis.		Duration.
Not Washed.		Washed.		Washed.*	Not Washed.	Washed.	Not Washed.	Washed.	
52†	54	53	58	57	48†	56	43†	49	
Percentage of total nitrogen in non-coagulable form.									
10.7	9.7	13.5	9.5	8.5	12.7	8.3	26.6	18.3	Control
15.5	18.6	35.5	23.6	19.3	20.0	18.5	39.4	41.1	1 day
18.2	27.8	57.6	38.5	24.1	24.9	65.1	51.1	48.5	3 days
19.8	27.8	67.5	49.7	29.6	30.8	75.7	54.3	61.0	5 days
19.9	29.1	70.1	54.2	37.9	32.1	79.4	60.4	82.3	8 days
13.4	11.6	14.0	8.9	7.4	14.5	7.2	22.1	18.1	Final Control
Phosphotungstate-filtrate nitrogen (monamino-acid).									
7.2	6.7	7.4	4.2	5.5	10.7	3.6	15.0	11.3	Control
67.3	69.3	54.8	44.5	64.7	81.1	43.0	56.5	51.7	
12.4	13.7	30.9	18.2	14.7	17.2	16.4	29.7	30.0	1 day
80.0	73.6	87.0	77.1	72.8	86.0	88.6	75.4	73.1	
14.1	21.4	49.8	31.8	19.9	22.6	54.7	37.2	39.3	3 days
77.5	77.0	86.6	82.6	82.5	90.8	84.2	72.8	81.0	
16.2	22.9	56.9	40.3	25.3	26.0	64.3	43.8	53.0	5 days
81.8	82.4	84.3	81.0	85.4	84.4	84.9	80.7	86.9	
16.9	23.9	57.8	46.2	32.3	27.2	70.1	46.9	71.9	8 days
84.9	82.7	80.1	85.2	85.2	84.7	88.3	77.6	87.3	
8.4	8.1	7.6	4.8	6.8	8.9	3.7	11.9	11.9	Final
63.0	70.0	54.1	54.3	90.5	75.4	51.5	53.8	66.0	Control
Ammonia nitrogen.									
0.74	0.49	0.59		0.79	0.80	0.85	2.08	1.10	Control
6.9	5.1	4.3		9.4	6.3	10.2	8.6	6.0	
1.38	0.90	1.41		1.05	1.46	1.50	4.4	2.20	1 day
8.8	4.8	4.0		5.4	7.3	8.1	10.6	5.3	
1.45	1.06	2.45		1.9	2.16	2.78	4.7	3.30	3 days
7.9	3.8	4.2		7.9	8.6	4.3	9.2	6.9	
1.63	1.60	2.52		1.6	2.19	3.25	5.8	3.99	5 days
8.2	5.1	3.8		5.4	7.1	4.3	9.6	6.5	
2.08	1.22	2.45		2.9	2.34	3.01	6.4	5.11	8 days
10.4	4.2	3.5		7.6	7.3	3.8	10.6	6.2	
0.66		0.67			1.02	0.85	2.2	1.43	Final
4.9		5.2			8.6	11.6	10.0	7.9	Control

* Washing incomplete.

† Distilled water used instead of neutral Ringer's solution.

Figures in upper left-hand corner show percentage of total nitrogen; those in lower right-hand corner, of non-coagulable nitrogen.

eight hour washed tissue (Dog 56) with a very extensive necrosis, showed the greatest increase, equivalent to 856 per cent. of the control. The increase of the four hour experiment (congestion and thrombosis) hardly equaled that of the washed normal.

Wherever water was employed in washing or in diluting, the autolysis was distinctly less than when neutral Ringer was used. (Compare Dogs 52 and 54.)

Concerning the rapidity of the autolysis, it may be noticed that, though the initial increase during the first day in the case of the unwashed tissues is but one half of that of the washed, it represents, as does also the increase of the washed, fifty per cent. of the total autolysis. On the third day, however, the autolysis has reached its maximum in the unwashed tissues, while the washed organs continue to increase until the eighth day, when the autolysis in their case is also apparently complete.

In the forty-eight hour lesions in which, histologically, the autolysis of the necrotic areas would appear to be at its height, we see that the autolytic processes *in vitro* were also very active. The increase at the end of the eighth day in the unwashed liver (Dog 48) was about 150 per cent., but after the removal of the inhibitory action of the blood (Dog 56) the increase rose to almost 900 per cent. The same thing is evident in the fifth day lesions but is not so pronounced.

The rapidity with which the autolysis reaches its maximum is of course dependent upon various factors. The attainment of the maximum signifies that the reaction velocities of the system, made up of substrat, hydrolytic agent and enzyme, have reached an equilibrium, caused, no doubt, by the non-removal of the products of autolysis. Since we must assume that the substrat and enzyme are the same in the normal tissues of both washed and unwashed organs, the varying factor must consist in the hydrolysis which, from the work of Wiener, seems undoubtedly due to the unneutralized acids formed during autolysis as first described by Magnus-Levy.[9] The acids which are formed in the normal metabolism of the cells are neutralized by the ammonia and excess of bases in the blood; hence

[9] Magnus-Levy, A., Ueber die Säurehildung bei der Autolyse der Leber, *Beit. z. chem. Physiol. u. Path.*, 1902, ii, 261.

autolysis does not occur in the living cell. As soon as the serum with its neutralizing power is removed, as in the washed organs or where the acids use up the excess of bases as in the center of a large area of necrosis, the conditions necessary for autolysis are present and hydrolysis of the substrat protoplasm takes place.

Phosphotungstate Filtrate Nitrogen (*Monamino-acids*).—The phosphotungstate precipitate has been disregarded here, for, as it consists of diamino-nitrogen it has been sufficiently covered in the autolysis experiments in connection with the study of the hexon bases.[10]

By far the major portion of the nitrogen in the filtrate is in the form of monamino-acids.[11] The table indicates the percentage of the fraction in terms of the total nitrogen of the tissue as well as of the total non-coagulable nitrogen. Our figures for the controls indicate that in the normal tissue, washed or unwashed, 4.2 to 7.4 per cent. of the total nitrogen is to be attributed to nitrogen not precipitable with phosphotungstic acid. Of the forty-eight hour lesions, that with the most marked diffuse necrosis (Dog 48) showed 10.7 per cent. of the total nitrogen in that form, while the other, of the focal type (Dog 56), had only 3.6 per cent. or slightly less than the lowest of the normal figures. Also, in the first of this pair, 81.1 per cent. of the non-coagulable nitrogen was in the form of monamino-acid while the other showed only 43.0, again somewhat less than normal.

These two experiments illustrate most decisively the point which Taylor's[12] results seem to indicate. That is, there is an absence of monamino-acids in pathological conditions of the liver accompanied by little or no necrosis, while in necrosis of the diffuse type both the monamino- and diamino-acids are present. We have elsewhere[13] suggested that the relation of circulatory disturbances to

[10] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[11] v. Drjewezki, A., Ueber den Einfluss der alkalischen Reaktion auf die autolytischen Vorgänge in der Leber, *Biochem. Zeit.,* 1906, i, 229.

[12] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskorper in der degenerirten Leber, *Zeit. f. physiol. Chem.,* 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications,* 1904, i (Path.), 43.

[13] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

the removal of the products of autolysis is an all-important factor. This is further supported by the two experiments under discussion which indicate that the organ with the focal lesions contained no more monamino-nitrogen than did the normal tissue. In this case the circulation was very slightly, if at all, impaired, and these acids, if they were formed, were removed immediately by the blood stream. In Experiment 48 the large necrotic areas, the centers of which were remote from circulatory fluids, held the acids as they were produced. That these substances are produced in autolysis of this type *in vivo* in large quantities is also indicated by the fact that 81.1 per cent. of the non-coagulable nitrogen of this liver was present in the fresh tissue as nitrogen non-precipitable with phosphotungstic acid. This value approaches that found in all the other cases after autolysis *in vitro* has proceeded for from one to three days.

The control figures of the five day necrosis, as shown by Experiments 43 and 49, also indicate a high percentage of the total nitrogen of the tissue as monamino-nitrogen. As, however, an unusually large amount of the total nitrogen occurs in non-coagulable form the percentage relation of the monamino acids to the latter is about normal. These lesions were very extensive but of the focal type and the cells at the fifth day were undergoing repair. Hence, although autolytic processes were going on in the tissue at that time, the products were removed as fast as they were formed and no increase in amount occurred in the organ.

The same differences in the velocity and degree of autolysis *in vitro* between the washed and unwashed organs are also very evident and require no discussion since they are due to the same causes. It is interesting to note that the nitrogen occurring as monamino-acids reaches at the end of the first day about 80 per cent. of the non-coagulable nitrogen and then although autolysis may greatly increase, their formation increases only in the same proportion. The exception to this is in case of Dog 48, already discussed, where the percentage was high in the tissue itself and remained at the same level during autolysis *in vitro*. This would seem to point to the fact that as far as monamino-acids are concerned their formation in autolysis *intra vitam* occurs in the same manner and by the same chemical processes as they do in autolysis *in vitro*.

v. Drjewezki found that at the end of seventy-two hours autolysis the monamino-acids took up about sixty per cent. of the total nitrogen of the tissue. This figure is somewhat higher than we obtained at this stage of autolysis but in some instances it was reached on the fifth or eighth day.

Ammonia.—As a result of the work of Loewi,[14] Jacoby,[15] Lang and others, considerable interest has become attached to the power of the surviving liver tissue to produce ammonia, especially in view of the current opinion as to the importance of this product, after it has been split off from the amino-acids, as a step in the formation of urea. This interest has been heightened by the appearance of ammonia in increased absolute and percentage amounts in the urine in certain hepatic disorders, thus apparently bringing these matters into correlation.

We have studied the question in two ways. First, in connection with the nitrogen partition of the autolysis now under discussion, and secondly, after the manner of the discoverer[15] of the ammonia-forming power of the liver. This latter part of the work will be discussed separately.

In the partition tables it is seen that although the ammonia content of the necrotic livers is greater than that of the normal it runs parallel with the increase in the amount of non-coagulable nitrogen. Hence the percentage figures show no regular increase or diminution though variations occur owing to the large limit of error dependent on the small amounts of ammonia formed. A comparison of a forty-eight hour necrotic liver (56), which offers an exception to the above statement, in that it shows a progressive diminution with a normal washed liver (53), is instructive. In 53 the normal percentage of ammonia of the non-coagulable nitrogen runs along at about 4 per cent. throughout the experiment. In 56, however, the control shows a high initial ammonia content (10.2), which on the third day dropped to that of the normal washed liver (53) and remained at that level to the end of the experiment.

[14] Loewi, O., Ueber das Harnstoffbildende Ferment der Leber, *Zeit. f. physiol. Chem.*, 1893, xxv, 511.

[15] Jacoby, M., Ueber die fermentative Eiweissspaltung und Ammoniakbildung in der Leber, *Zeit. f. physiol. Chem.*, 1900, xxx, 149.

We explain this variation in the ammonia formation by the assumption that this tissue contained *intra vitam,* as the result of the necrosis, proportionately larger amounts of ammonia liberating compounds than the normal. As, however, the autolysis proceeded less of these products were formed in relation to the non-coagulable nitrogen than was the case in the autolysis of the normal. Hence the percentage figure dropped. Or if we allow for the initial difference we find that the percentage increase is the same in both cases.

The great increase in the amount of non-coagulable nitrogen which occurred between the first and third day (18.5 to 65.1 per cent.) in Dog 56 could not have included the formation of ammonia compounds, since the increase of these latter bodies in relation to the total nitrogen was so slight that there occurred an actual percentage decrease in relation to the non-coagulable nitrogen.

All this would seem to indicate that the production of ammonia which occurs in the autolysis of the liver *in vitro* is the result of a decomposition of coagulable nitrogen in the cell. That is deamidization of the amino-acids and the splitting of urea does not take place to any greater extent in the necrotic tissue undergoing autolysis than it does in the normal.

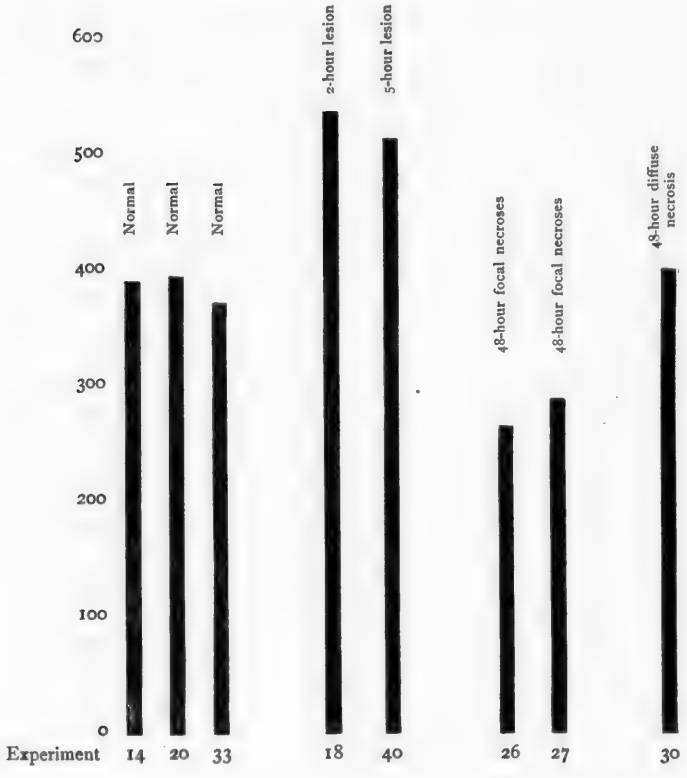
Separate Ammonia Determinations.—These experiments were some of the earlier ones performed and were carried out after the fashion of the investigations reported by Jacoby. A weighed amount, 200 grams, of the finely divided fresh liver tissue was made up to 600 cubic centimeters with distilled water and the well-mixed fluid divided into twelve equal parts. Four of these samples were sterilized immediately for controls. Two were analyzed at once as initial controls, while two, for the purpose of final controls, were placed with the remainder of the portions in the thermostat. At varying periods, duplicate samples were removed for analysis. The ammonia was determined according to a modification of Shaffer's method for the urine. The accompanying diagram indicates in a schematic way the results of the experiments.

It is somewhat difficult to make comparison of these results with those just reported in the partition series since we have no determination of the amount of non-coagulable nitrogen at the various

TABLE II.

Ammonia.

Percentage increase at the end of ten days.



periods; hence the figures represent only the percentage increase of ammonia nitrogen based on its amount in the control sample. Although Jacoby gives in his tables only the amount of ammonia nitrogen formed without taking into consideration the question of dry solids and nitrogen of the dry residue, a recalculation of his figures from average results of our normal livers indicates that more ammonia nitrogen was formed in our experiments than in his. On the other hand, however, our figures are not as high as those re-

ported by Soetbeer.[16] In opposition to the partition series, our figures indicate a larger amount of ammonium compounds in the initial control sample of the normal tissue than in those with varying degrees of necrosis and degeneration. This seeming anomaly we are unable to understand or explain. As, however, the percentage increase figures upon which the diagram is calculated are based upon the initial control as zero, the variable and anomalous control factor is excluded.

Considered in this way it will be seen that the three normals showed an increase of ammonia-nitrogen over the control of from 378 to 396 per cent., an agreement which serves well as a basis for comparison of the experiments on necrotic and degenerated tissue. In the case of the focal necroses the increase is less than the normal, amounting only to 265 to 290 per cent. On the other hand, the diffusely necrotic tissue evidenced the same power to produce ammonia-forming compounds as the normal. In the two samples of congestion and thrombosis, the lesion being of two to five hours' duration, more ammonia was produced than in the normal. These results would seem to indicate that during the initial stage of the process when the liver is intensely congested an increase in ammonia output must occur. This, however, is not supported by our metabolism experiments.[17]

Uric Acid.—The investigation concerning uric acid has yielded results of not sufficient interest for presentation in a table. The only point of importance is that a gradual diminution occurs which ceases on the third day. Since in the autolysis of uric acid ammonia is formed, this factor must influence to a slight extent the increase in ammonia observed in the partition experiments.

Arginase.—In the endeavor to explain the results in connection with the hexon bases, reported in the first paper of this series, a few experiments were conducted in the attempt to prepare from normal and necrotic livers an active substance, according to the

[16] Soetbeer, F., Ueber einen Fall von akuten Degeneration des Leberparenchyms, *Arch. f. exper. Path. u. Pharm.*, 1903, l, 294.

[17] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

method of Kossel and Dakin,[18] which would hydrolyze arginin into ornithin and urea. Preparations were made by both the ammonium sulphate and acetic acid-ether methods outlined by these investigators and solutions of these were added in aliquot parts to an arginin solution of known strength. The determinations were made sometimes upon the phosphotungstic precipitate, sometimes upon the filtrate from this and once upon both. The aliquots were allowed to autolyze for one, three and five days and controls were done at the beginning and at the end of the experiment.

TABLE III.

Arginase.

Experiment.	Lesion.	Method of Preparation.	Estimation on Phosphotungstate.	c.c. N/10 Acid.			
				Control.	1 day.	3 days.	5 days.
15	Normal.	¾ Saturation $(NH_4)_2SO_4$ precipitate.	filtrate.	1.6	2.65	2.75	2.65
15	Normal.	ditto.	filtrate.	4.55	5.95	6.25	6.80
15	Normal.	ditto.	precipitate.	5.75	4.45	4.25	3.65
19	Normal.	ditto.	precipitate.	4.50	3.40	5.00	5.65
42	Normal.	Extraction acetic acid.	filtrate.	5.20	4.45	4.15	4.30
18	2 hours.	Complete saturation $(NH_4)_2SO_4$ precipitate.	precipitate.	11.45	12.10	12.40	9.90
40	5 hours.	Extraction acetic acid.	filtrate.	5.40	4.65	5.30	4.40
16	Focal necroses.	Complete saturation $(NH_4)_2SO_4$ precipitate.	precipitate.	6.75	6.70	6.55	5.70
28	Diffuse necrosis.	ditto.	precipitate.	6.10	6.90	6.95	6.10
28	Diffuse necrosis.	Extraction dilute HCl.	precipitate.	8.05	9.80	8.45	8.60

Our results in regard to the normal liver agree with those of Kossel and Dakin. The preparations from necrotic livers gave negative or doubtful results, thus affording valuable confirmatory evidence of the position which we assumed as a result of our work upon the hexon bases, namely, that in extreme diffuse necrosis where large areas remote from the circulation are undergoing necrosis

[18] Kossel, A. and Dakin, H. D., Ueber die Arginase, *Zeit. f. physiol. Chem.*, 1904, xli, 321. Weitere Untersuchungen ueber fermentative Harnstoffbildung, *ibid.*, 1904, xlii, 181.

there occurs a marked increase in hexon base content of the tissue. In such areas, evidently, the arginin is not split up to any noticeable extent. The experiments of Wakeman[19] do not show the presence of arginin to the marked extent which the author claims, as we have explained in our discussion of the hexon bases.[20]

The table shows that of the ten preparations made from seven different livers, three normal, two necrotic and two with thrombosis, only one, the normal (15), showed any activity. In the others either no results were obtained or were so irregular that the experiments may be considered as negative.

In this one experiment with normal liver in which the precipitate obtained by complete saturation of the three-quarter saturated filtrate with ammonium sulphate was employed, the nitrogen of the filtrate from the phosphotungstic precipitate showed a gradual increase presumably due to the autolysis of the arginin added. These results would indicate that an active arginase can be obtained only from normal tissue. This is in complete accord with the results reported in the paper on the hexon bases in which it is shown that during the autolysis of necrotic liver tissue an increase in the hexon base content of the cell occurs.

Leucin and Tyrosin.—In view of the well-recognized presence at times of monamino-acids in the urine of individuals suffering with hepatic disorders, particularly acute yellow atrophy, and of the varying results reported by the different observers in regard to the question of the presence of these compounds in the liver tissue, it seemed advisable to examine the urine and the liver of the animals under observation as to the presence of leucin and tyrosin.

It would appear to be unnecessary for us to enter into a discussion of the older literature concerning the variation in the results as to the presence or absence of leucin and tyrosin in the urine under many pathological conditions. This subject has been well discussed by Ewing and Wolf,[21] who conclude that the differences observed

[19] Wakeman, A. J., On the Hexon Bases of Liver Tissue under Normal and Certain Pathological Conditions, *Jour. of Exper. Med.*, 1905, vii, 292.

[20] See first paper of this series, "Hexon Bases" in this number of the *Journal.*

[21] Ewing, J. and Wolf, C. G. L., The Clinical Significance of the Urinary Nitrogen, *Amer. Jour. of the Med. Sciences*, 1906, cxxxi, 751.

are most probably due to faulty methods of technique and of confirmation. Of the recent work, to which this criticism cannot properly be applied is that of Taylor,[22] who found these monamino-acids present in the liver of acute yellow atrophy as well as in that of probable chloroform poisoning. In both instances, supposedly, necrosis of varying degree had taken place. Wells[23] in a preliminary communication confirms these results for acute yellow atrophy. On the other hand, however, in other conditions to which he gives the general term of " degeneration," Taylor failed to find these substances. Again leucin and tyrosin usually appear in the urine of persons or animals poisoned with phosphorus and this fact has been associated with the occurrence of the well-known hepatic changes, chiefly fatty infiltration, which are known to occur in this condition.

The recent method devised by Fischer and Bergell, in which β-naphthalin sulphochloride is employed, and Abderhalden and Barker's modification of Fischer's esterification method are so time-consuming that we decided that for the purpose in view, the simpler methods were of sufficient accuracy to warrant their use. Ewing and Wolf in the paper mentioned above criticize severely the lead acetate method, originally employed by Frerichs and Städeler. They claim that the microscopic demonstration of leucin and tyrosin by this procedure is unreliable and the crystals supposed to be leucin may be in reality urates or urea. We have used a modification of the lead acetate method in which after the removal of the excess of lead by means of hydrogen sulphide the filtrate is evaporated to dryness and the residue extracted with several portions of absolute alcohol to remove the urea, after which it is treated with repeated portions of ammoniacal absolute alcohol. The united extracts are allowed to evaporate almost to dryness, when characteristic crystals appear, if leucin or tyrosin is present in the original material. When sufficient quantities were present these microscopic findings were controlled by the usual chemical tests. We feel reas-

[22] Taylor, A. E., Ueber das Vorkommen von Spaltungsprodukten der Eiweisskorper in der degenerirten Leber, *Zeit. f. physiol. Chem.*, 1902, xxxiv, 580. On the Occurrence of Amino-acids in Degenerated Tissue, *Univ. of California Publications*, 1904, i (Path.), 43.

[23] Wells, H. G., The Composition of the Liver in Acute Yellow Atrophy. Communication read at the first meeting of the Amer. Soc. of Biol. Chemists, Washington, May 8, 1907.

onably sure that the substances upon which we have based the following results were leucin and tyrosin.

TABLE IV.

Leucin and Tyrosin in the Urine.

Experiment.	Lesion.	Leucin.	Tyrosin.	Urine of
2	No necroses	—	+	4th day
34	No necroses	+++	+	1st and 2d day
1	Focal necroses	+	+	1st day
5	Focal necroses	—	+	1st day
23	Focal necroses	++	+	1st and 2d day
32	Diffuse necrosis	—	+	1st day
48	Diffuse necrosis	—	++	1st and 2d day
51	Diffuse necrosis	—	++	2d and 3d day

TABLE V.

Leucin, Tyrosin and Proteoses in the Liver.

Experiment.	Lesion.	Leucin.	Tyrosin.	Proteoses.	Age of Lesion.
16	Focal necroses	—	+	+	48 hour
32	Diffuse necrosis	—	+	+	26 hour
48	Diffuse necrosis	—	++	+	48 hour
51	Diffuse necrosis	—	++	+	48 hour

The table giving the results of the examinations of the urine shows that there is no regularity in the occurrence of these compounds. The type of the lesion has apparently no relation to the amount eliminated and the results presented justify the general concensus of current opinion that the appearance of these compounds is not to be regarded as pathognomonic of any one condition such as acute yellow atrophy or phosphorus or chloroform poisoning. In addition to the positive results presented in the table the urine of nine other animals was examined with negative results. In five of these the liver showed necroses, in four none.

The results of the examination of the liver substance point to the occurrence of tyrosin in larger amounts when the lesion was most pronounced; thus in each of three livers with diffuse necrosis it was present, but in only one of the five examples of focal necroses did it occur. A normal liver and also one with degeneration but no necroses were likewise negative. In no condition did we find leucin. In four livers with extensive necrosis proteoses were found in considerable quantities while a normal liver yielded none. All of

this is in agreement with the variable results of Taylor mentioned elsewhere.

It is evident, therefore, that leucin and tyrosin may be formed during the autolysis of the hepatic tissue, but their appearance in the urine or detection in the liver is dependent upon the condition of the hepatic cells not involved in the lesion. If these cells can take care of large quantities of monamino-acids carried to them normally by the portal vein we see no reason why, if they are present in sufficient numbers and properly functionating, that they should not react in the same way with the same acids formed during the autolysis. The appearance of these monamino-acids under any condition then would depend upon the quantitative relation of the necrosis to the actively functionating cells which are unaffected by the lesion.

Of considerable interest in connection with the finding by Salkowski[24] in the urine of various pathological conditions, more particularly a case of yellow atrophy, of an increased amount of nitrogen precipitable by alcohol, is the fact that although in the normal liver the residue remaining after the extraction with absolute alcohol and ammoniacal alcohol is small in amount, in the case of the necrotic tissues this amount is markedly increased. We have examined the residue as to its character and find that it consists mainly of proteoses. The removal of these compounds by way of the blood-stream would cause an increase in the urine of undetermined nitrogen usually ascribed to amino-acids. This occurrence explains those conditions characterized by a high rest-nitrogen without the presence of monamino-acids.

SUMMARY.

1. The presence of blood serum has a decided inhibitory effect on autolysis. Thus in the normal unwashed organs the non-coagulable nitrogen increase was 100 to 300 per cent., while in the washed it amounted to 450 per cent. The washed necrotic livers showed an increase of from 600 to 850 per cent., while that of the unwashed necrotic was only slightly above the normal unwashed.

2. While the initial amount of non-coagulable nitrogen varies it is greater in those livers showing the more extensive forms of

[24] Salkowski, E., Zur Kenntnis der Alkoholunlöslichen bzw. kolloidalen Stickstoffsubstanzen im Harn, *Berl. klin. Woch.*, 1905, xlii, 1581, 1618.

necrosis. The final amount of autolysis is also greatest in livers of this type. As regards the rate of autolysis fifty per cent. of the total occurs in the first day in the normal and in all types of lesions both washed and unwashed. The maximum is usually reached on the third day in the unwashed, while in the washed there is a continued increase to the eighth day. At this time in the necrotic livers about two to three times as much of the total nitrogen is in the form of non-coagulable nitrogen as in the normal.

3. In the necrotic tissue the initial controls show the content of monamino-acids, with one exception, to be practically doubled. In the washed necrotic the final amount is seventy per cent. of the total nitrogen against forty-six to fifty-seven per cent. in the washed normal. In all cases the monamino-acid nitrogen runs parallel to the nitrogen in non-coagulable form, but in relation to the total nitrogen it shows a greater increase in the washed than in the unwashed organs.

4. The ammonia production in the necrotic livers as shown by the partition experiments is greater than that in the normal and this increase corresponds to that of the non-coagulable nitrogen. In the experiments concerning the absolute production of ammonia in the presence of serum a greater amount was produced in the two and five hours' lesions than in the normal livers. On the other hand, the forty-eight hour diffuse necrosis equaled the normal and the focal fell below.

5. Arginase was obtained from normal but could not be isolated from necrotic livers.

6. No constant relation could be demonstrated between the anatomical lesion in the liver and the presence of leucin and tyrosin in the urine. Leucin was found occasionally in the urine, but none in the liver. On the other hand, tyrosin was constantly present in livers with diffuse but rarely in those with focal necrosis. In the instances of diffuse necrosis in which the liver and urine of the same animal were examined tyrosin was found in both.

7. The presence of large amounts of proteoses in the necrotic liver indicates that the elimination of these substances (colloidal nitrogen of Salkowski) under such circumstances may account for a part of the total nitrogen of the urine usually attributed to the monamino-acids.

EXPERIMENTAL LIVER NECROSIS; III. NITROGENOUS METABOLISM.[1]

By RICHARD M. PEARCE, M.D.,
Professor of Pathology and Bacteriology,

AND

HOLMES C. JACKSON, Ph.D.,
Adjunct Professor of Physiological Chemistry,
Albany Medical College.

(*From the Bender Laboratory, Albany, N. Y.*)

We present here the results of experiments carried out upon dogs in which the general state of the nitrogenous metabolism of the animals was studied before and after the injection of hæmotoxic immune sera. As has been stated elsewhere[2] the important organic lesion produced by such sera is a hepatic necrosis either focal or diffuse, according to the amount and strength of the serum employed. Such an experimental lesion seemed peculiarly adapted to the study of the function of the liver in general metabolism and of certain peculiar metabolic derangements which have an analogy to those noted in eclampsia, chloroform poisoning, vomiting of pregnancy and acute yellow atrophy. It must be clearly borne in mind, however, that this experimental method of producing hepatic necrosis allows of the infliction upon the liver of a single injury, in point of time, which if not followed by death is rapidly repaired and which is almost without other disturbing factors. Hence metabolic variations may be transient and last but one or two days, and for this reason the resulting disturbance is not comparable to that produced by a continually acting cause with progressive lesion.

The experiments were carried out upon dogs kept in the usual

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Read before the American Association of Pathologists and Bacteriologists, Washington, May 7, 1907. Received for publication July 2, 1907.

[2] See first paper of this series, "The Hexon Bases," in this number of the *Journal*.

well-ventilated metabolism cages and fed upon a purin-free diet of casein, cracker dust and lard. The amounts of the three dietary constituents varied for the different animals. They were calorifically sufficient and so regulated that the animal finally came into nitrogen equilibrium. The dogs were catheterized at the end of each twenty-four hour period and the urine thus obtained, added to that voided naturally. This was at all times carefully preserved from changes of any kind until the analytic work was completed. To the total twenty-four elimination was added distilled water to make the volume up to 800 cubic centimeters. When hæmoglobinuria or albuminuria caused the appearance of proteid in the urine this was removed by heat and acetic acid, the coagulum being thoroughly boiled out with water and the washings added to the urine.

Upon these twenty-four hour samples the following determinations were made: total nitrogen by the Kjeldahl-Gunning method; ammonia by the Shaffer method; urea by the Mörner-Sjoquist method; uric acid by the Hopkins-Folin method and creatinin by Folin's colorimetric adaptation of Weyl's qualitative test. The difference between the sum of these various factors and the total nitrogen is given as rest or undetermined nitrogen. Kynurenic acid was also looked for qualitatively in order to obtain evidence as to variations in the output of this substance.

The general procedure in these experiments was to place the animal upon nitrogen equilibrium and to conduct control determinations for a period of three days, after which the animal was injected with either normal or toxic sera and the experiment allowed to continue until death ensued or the metabolism had regained its normal level as shown by the control period. It is obvious that in experiments of this character it becomes extremely difficult, if not impossible, owing to variance in the toxicity of the sera and the susceptibility of the animals, to regulate the severity of the lesion, especially as the degree of the latter can be determined only by post-mortem examination. This fact prohibits the production in any two experiments, no matter how carefully planned, of absolutely comparable pathological conditions. This difficulty is brought out by the fact that although each new lot of serum was tested for lethal action on dogs of approximately the same weight and conditions as

those prepared for the experiment, only six out of twelve of the latter survived the first twenty-four period. These six, with a control dog receiving normal serum, constitute the experiments on which this communication is based.

These may be divided into four groups: one injected with normal serum in which no histological change took place; one with a weak toxic serum which caused no necrosis, but an extensive granular and vacuolar degeneration; four with toxic sera causing more or less extensive focal necroses and one with toxic serum producing diffuse necrosis.

TABLE I.

(*Dog 18, normal serum.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Nov. 10	6.51	4.99 76.8	0.556 8.5	0.018 0.28	0.677 10.4	0.269 4.0	
11	6.09	4.81 78.9	0.492 8.1	0.013 0.21	0.633 10.4	0.142 2.4	
12	6.52	5.25 80.5	0.493 7.6	0.020 0.30	0.607 9.3	0.150 2.3	Injected 12 M. Dose 1: 738. Vomited.
13	6.60	5.06 76.6	0.510 7.7	0.017 0.26	0.576 8.7	0.437 6.7	
14	6.06	4.72 77.8	0.482 8.0	0.021 0.34	0.539 8.9	0.298 5.0	

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

Table I shows the result of the injection of normal serum. With this may be compared also Tables VI and VII, in which it is seen that the toxic serum was preceded by an injection of normal serum. In two of these the dose[3] of the normal serum was greater than that of any injection of toxic serum. It will be seen that it was practically without effect although in Dog 48 (Table VI) a slight increase in the output of total nitrogen was evident. A consideration of the nitrogen partition indicates, however, that this increase is mainly at the expense of the rest-nitrogen and is to be explained by traces of foreign proteid of the rabbit's serum injected, too small to be removed by the ordinary methods. We possess, there-

[3] The figures representing dosage, for example 1:600, indicate that the dose was in the proportion of one cubic centimeter of serum to 600 grams of body weight.

fore, a series of controls upon which to base our conclusions concerning the effect of the toxic sera.

Table II gives the figures obtained as the result of injecting a weak serum,[4] which caused extensive hepatic degeneration of the granular and vacuolar type but no necrosis.

TABLE II.

(*Dog 12, Degeneration, No Necrosis.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Oct. 15	4.33	3.67 84.5	0.157 3.6	0.006 0.14	0.302 7.0	0.195 4.76	
16	3.77	3.24 85.7	0.131 3.5	0.005 0.13	0.285 7.6	0.109 3.07	
17	3.76	3.29 87.3	0.120 3.1	0.004 0.13	0.284 7.6	0.062 1.87	
18	3.16	2.72 87.1	0.104 3.3	0.004 0.13	0.241 7.6	0.060 1.87	
19	4.23	3.56 84.1	0.090 2.1	0.004 0.09	0.315 75.	0.261 6.21	Injected 4.30 P.M; weak toxic serum; dose 1:715.
20	3.37	2.66 78.9	0.165 4.9	0.003 0.09	0.288 8.5	0.254 7.66	
21	3.19	2.69 84.3	0.161 5.1	0.006 0.19	0.268 8.4	0.065 2.01	
22	3.02	2.60 86.1	0.122 4.0	0.002 0.06	0 260 8.7	0.036 1.14	Killed.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

It is seen that a slight but transient rise in the total nitrogen occurred. A small part of this increase is attributable to the foreign proteid injected, with a corresponding rise in the undetermined nitrogen, both in absolute and percentage amounts. The absolute amount of urea nitrogen increased during the first twenty-four hours after injection but not in sufficient quantities to keep pace with the increase in total nitrogen, hence a percentage decrease occurred. The following day both the percentage and absolute amounts diminished markedly to be followed on the succeeding days by return to the normal percentage of urea output. The absolute quantity remained low since the total nitrogen did not return to nor-

[4] The serum of a rabbit which was not bled until six weeks after immunization against dog's blood. Sera obtained so long after injection frequently show diminished hæmagglutinative and hæmolytic power.

mal. The ammonia output during the twenty-four hours succeeding the injection suffered a decided diminution both in percentage and absolute figures. This decrease was exceedingly transient, since on the succeeding days the reverse occurred and both the absolute and percentage figures went much above the normal. The uric acid and creatinin showed no change but the rest-nitrogen increased considerably on the first and second days following the injection.

The reaction[6] for kynurenic acid was positive on the day subsequent to the injection and passed off gradually. This would indicate an increase in proteid destruction.[7]

Of most importance is the diminution in the percentage of the total nitrogen eliminated as urea associated with a somewhat corre-

TABLE III.

(*Dog 25, Focal Necroses.*)

Date.	Total Nitrogen.	Urea.*		Ammonia.		Uric Acid.[5]	Creatinin.		Undetermined Nitrogen.		Notes.
Dec. 2	4.77	3.55	76.5	0.443	9.1		0.405	8.4	0.372	6.0	
3	5.42	4.39	81.0	0.507	9.4		0.362	6.7	0.161	3.3	
4	5.24	4.11	78.4	0.440	8.4		0.298	5.7	0.392	7.5	
5	4.97	3.86	77.6	0.421	8.5		0.335	6.7	0.354	7.2	
6	6.02	4.68	77.4	0.496	8.2		0.362	6.0	0.482	8.4	Injection 2:30 P.M. toxic serum dose 1 : 1000. Vomited.
7	5.85	4.77	81.5	0.443	7.6		0.351	6.0	0.286	4.9	Hburia.
8	5.92	4.95	83.6	0.313	5.3		0.323	5.3	0.334	5.8	Hburia.
9	9.33	7.66	82.1	0.587	6.3		0.430	4.6	0.653	7.0	Hburia.
10	6.46	5.40	83.5	0.376	5.8		0.360	5.6	0.324	5.1	
11	8.15	7.16	87.8	0.404	4.9		0.368	4.5	0.218	2.8	
12	8.22	7.07	86.0	0.461	5.6		0.366	4.5	0.323	3.9	No food taken; killed.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

[5] Amounts so small that they were not calculated.

[6] Treatment of the urine with bromine water.

[7] Mendel, L. B. and Jackson, H. C., On the Excretion of Kynurenic Acid, *Amer. Jour. of Physiol.*, 1898, ii, 1.

TABLE IV.

(*Dog 43, Focal Necroses.*)

Date.	Total Nitrogen.	Urea.*		Ammonia.		Uric Acid.		Creatinin.		Undetermined Nitrogen.		Notes.
Feb. 2	7.74	6.31	81.5	0.444	5.7	0.018	0.23	0.417	5.4	0.551	7.2	
3												Fæces mixed with urine.
4	6.95	5.50	79.1	0.406	5.8	0.013	0.17	0.338	4.9	0.693	10.0	
5	6.45	5.37	83.2	0.335	5.2	0.015	0.23	0.280	4.3	0.450	7.1	Injected 10:30 A.M. toxic serum dose 1 : 1738. Vomited.
6	9.91	8.48	85.5	0.478	4.8	0.073	0.73	0.343	3.5	0.836	8.5	Injected 3 P.M. toxic serum; dose 1 : 1200.
7	10.56	8.88	84.1	0.578	5.5	0.045	0.42	0.405	3.8	0.652	6.2	
8	7.65	6.31	82.5	0.479	6.2	0.009	0.12	0.386	5.0	0.466	6.2	
9												Urine lost.
10	4.85	3.84	79.2	0.288	5.9	0.007	0.14	0.341	7.2	0.374	7.6	Killed.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

sponding increase in the percentage output of ammonia and rest-nitrogen. This is the urinary picture which recently has been described as associated with the hepatic disorder supposed to underly the symptoms of chloroform poisoning, toxæmia of pregnancy and like conditions and which will be discussed more in detail after our results have been completely given.

Tables III, IV, V, VI and VII present the results of the experiments in which a true necrosis, either focal or diffuse, was obtained.

In these experiments the injection of the toxic sera[8] was always quickly followed by a more or less marked increase in the elimination of total nitrogen which *persisted* for several days. During the first or second twenty-four hour period, after injection, occurred a slight increase in the percentage of urea nitrogen (three to five per cent.) which was followed on the succeeding days by a drop to normal, and in one experiment below normal. The ammonia nitro-

[8] In three of these experiments the first injection was followed by a second after a varying interval. This fact renders the figures after the time of the second injection less comparable.

gen percentage of the total nitrogen diminished gradually after the injection and reached its lowest point about the second or third day, after which, in some cases, as in Dogs 49 and 43, it returned to normal; in others, as in Dogs 25, 45 and 48, it remained low. The more advanced the repair at the time of death, the nearer the percentage of ammonia nitrogen had returned to the normal.

The uric acid nitrogen suffered a marked, though transient, increase.[9] In the three experiments where successive injections were given, the second injection in each instance caused an increase of uric acid on the following day after which it returned to normal.

The absolute creatinin nitrogen output was noticeably augmented after injection; this increase, however, was not quite in the same proportion as the total nitrogen, hence the creatinin nitrogen per cent. of the total tended at times to show a slight diminution.

TABLE V.

(*Dog 45, Focal Necroses.*)

Date.	Total Nitrogen.	Urea.*		Ammonia.		Uric Acid.		Creatinin.		Undetermined Nitrogen.		Notes.
Feb. 3	6.70	5.50	82.1	0.342	5.1	0.014	0.21	0.274	4.1	0.570	7.5	
4	6.57	5.37	81.7	0.491	7.5	0.013	0.20	0.296	4.5	0.400	6.1	
5	6.43	5.05	78.5	0.452	7.0	0.012	0.18	0.274	4.1	0.642	10.2	
6	6.52	5.33	81.7	0.455	7.0	0.013	0.20	0.265	4.1	0.457	7.0	Injected 10 A. M. toxic serum; dose 1 : 1500
7	8.99	7.51	83.5	0.560	6.2	0.105	1.17	0.359	4.0	0.456	5.1	
8	7.97	6.25	78.4	0.524	6.6	0.010	0.13	0.300	3.9	0.886	11.0	Injected 10 A. M. toxic serum; dose 1 : 1000
9	5.30	4.01	75.6	0.441	8.3	0.055	1.04	0.298	5.7	0.928	9.4	
10	10.30	8.20	79.6	0.646	6.3	0.030	0.28	0.349	3.4	1.075	10.4	
11	8.37	6.71	80.2	0.598	7.1	0.021	0.25	0.313	3.7	0.728	8.8	
12	7.45	5.99	80.0	0.447	6.0	0.009	0.12	0.326	4.5	0.678	9.4	Killed

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

[9] For a detailed discussion of this subject see fourth paper of this series, "Nuclein Metabolism," in this number of the *Journal.*

TABLE VI.

(*Dog 48, Diffuse Necrosis.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Feb. 19	6.80	5.90 / 86.7	0.374 / 5.5	0.018 / 0.26	0.373 / 5.5	0.135 / 2.0	
20	6.55	5.53 / 84.4	0.360 / 5.5	0.018 / 0.27	0.364 / 5.6	0.278 / 4.2	
21	6.38	5.41 / 84.8	0.358 / 5.6	0.024 / 0.37	0.326 / 5.2	0.228 / 4.0	Injected 5 P. M. normal serum; dose 1:600.
22	7.00	5.76 / 82.3	0.406 / 5.8	0.024 / 0.34	0.338 / 4.8	0.472 / 6.8	
23	6.53	5.50 / 84.2	0.376 / 5.8	0.021 / 0.32	0.326 / 5.0	0.307 / 4.7	
24	6.10	5.00 / 82.0	0.400 / 6.6	0.018 / 0.30	0.314 / 5.1	0.368 / 6.0	Injected 10 A. M. toxic serum; dose 1:1155.
25				0.036			Vomitus mixed with urine.
26	8.78	7.58 / 86.3	0.365 / 4.1	0.018 / 0.20	0.345 / 4.0	0.472 / 5.4	
27	9.11	6.80 (?) / 76.4	0.551 / 6.0	0.018 / 0.20	0.349 / 3.8	1.392 (?) / 13.6	Injected 10 A. M. toxic serum; dose 1:600. Vomited.
28	11.12	9.24 / 83.1	0.418 / 3.8	0.130 / 1.17			Hburia. Vomiting; refused food.
Mar. 1				0.083			Died.

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

The undetermined or rest-nitrogen which in a general way may be said to indicate the output of amino-acids, polypeptids or proteose-like bodies[10] also underwent a decided increase after injection.

An increase in kynurenic acid elimination after injection was noticed at times, but this was slight at the best and in no way corresponds to the increase which occurs after the administration of phosphorus, phlorhizin and large quantities of meat. In these latter instances Mendel and Jackson[11] showed that the increased kynurenic acid output was associated with augmented endogenous

[10] Salkowski, E., Zur Kenntnis der Alkohlolunlöslichen bzw. colloidalen Stickstoffsubstanzen im Harn, *Berl. klin. Woch.*, 1905, xlii, 1581, 1618.

[11] Mendel, L. B. and Jackson, H. C., On the Excretion of Kynurenic Acid, *Amer. Jour. of Physiol.*, 1898, ii, 1.

or exogenous destruction of proteid material containing the tyrosin nucleus.

As has already been stated, we believe that the effect of the blood changes produced by the serum is directed almost entirely upon the liver and represents a single attack upon this organ. Two coincident conditions, which however do not affect the results in any manner, require perhaps a brief notice. In the first place, vomiting usually occurs and persists for a short time, five to ten minutes, after the injection. The feeding and injection were so arranged that nothing was lost in this manner and as the vomiting also occurred when normal serum was used, with no apparent effect on the metabolism, we believe this factor may be disregarded. It has occasionally, however, caused the loss, on account of admixture of vomitus, of a day's urine.

TABLE VII.

(*Dog 49, Focal Necroses.*)

Date.	Total Nitrogen.	Urea.*	Ammonia.	Uric Acid.	Creatinin.	Undetermined Nitrogen.	Notes.
Feb. 22	5.24	4.33 / 82.6	0.366 / 7.0	0.021 / 0.40	0.215 / 4.1	0.308 / 5.9	
23	5.48	lost	0.320 / 5.9	0.018 / 0.33	0.218 / 4.0		
24	5.46	4.41 / 80.8	0.345 / 6.3	0.021 / 0.38	0.208 / 3.8	0.476 / 8.7	Injected 10 A. M. normal serum; dose 1 : 600
25				0.018			Vomitus mixed with urine
26	5.25	4.34 / 82.7	0.297 / 5.7	0.021 / 0.40	0.194 / 3.7	0.398 / 7.5	Trace albumin
27	5.63	4.77 / 84.8	0.320 / 5.7	0.022 / 0.39	0.204 / 3.6	0.314 / 5.5	Injected 10 A. M. toxic serum; dose 1 : 628. Vomited
28	7.07	6.07 / 85.6	0.245 / 3.5	0.014 / 0.20	0.189 / 2.7	0.452 / 8.0	Hburia
Mar. 1	9.14	7.56 / 82.7	0.270 / 2.8	0.029 / 0.32	0.225 / 2.5	1.056 / 11.7	Hburia
2	8.45	6.98 / 82.6	0.428 / 5.1	0.017 / 0.20	0.221 / 2.6	0.804 / 9.5	Hburia
3	6.97	6.04 / 86.6	0.370 / 5.3	0.022 / 0.31	0.214 / 3.1	0.324 / 4.7	Hburia
4	5.70	4.92 / 86.3	0.366 / 6.4	0.020 / 0.35	0.196 / 3.4	0.198 / 3.6	Hburia. Killed

* Figures in upper left-hand corner represent grams nitrogen; those in lower right-hand corner, percentage of total nitrogen.

In the second place hæmoglobinuria, sometimes but not always, makes its appearance after twenty-four hours. This condition, however, could not have produced the changes in the general metabolism which we have described since in two of the experiments (43 and 45), where no hæmoglobin or bile appeared in the urine, the results were the same as in those showing a well-marked hæmoglobinuria. This agrees with the observations of Samuely[12] who found in experimental anæmia, produced by means of pyrodin, that the appearance of hæmoglobin or bile in the urine stood in no direct relationship to the changes which took place during the anæmia. On the other hand, Andrea[13] in a series of experiments, in which various hæmolytic substances (phenylhydrazin, pyrogallol, p-phenylendiamin, glycerin) were administered to rabbits, has found an increase of urea after the initial injection, but a decrease of one third after subsequent injections. The increase he explains by destruction of hæmoglobin and the decrease as due to impaired hepatic function.

Another possibility, however, must also be considered. This is that the temporary anæmia which is simultaneously produced as the result of the primary action of the serum on the red cells may originate changes in oxidization capable of accounting for some of the results. If the anæmia were general in character and of the type which occasions a greatly diminished oxidative power throughout the body, such as is noticed after carbon monoxide poisoning, then we should expect to find, among other disturbances, the elimination of incompletely oxidized products of catabolism, such as lactic acid. We have searched for the appearance of this substance in the urines of five of the animals showing necrosis, but have failed to find it. This, with other facts, appear to justify the exclusion of the factor of diminished oxidation.

In this connection it is of considerable interest to note also that in the type of anæmia produced by Samuely the power of the body to oxidize aromatic compounds, such as phenylalanin and cystein,

[12] Samuely, F., Stoffwechseluntersuchungen bei experimenteller Anämie, *Deut. Arch. f. klin. Med.*, 1907, lxxxix, 220.

[13] Andrea, P., Influenca della sostanze emolitiche sulle funzioni ureogenetica ed antitossica del fegato, *Arch. Int. de Pharmacodyn. et de Therapie*, 1905, xiv, 389.

was somewhat decreased, but that the metabolism in regard to the fatty amino-acids was absolutely unchanged.

Upon the whole, therefore, it seems justifiable to designate, as the main causative factor in the production of the results obtained, the necrotic lesions more or less diffusely distributed throughout the liver.

Several attempts have been made to study the influence of hepatic necrosis upon the metabolism. Jacoby[14] was the first to study the effect of tying off the vessels supplying certain lobes of the liver. Unfortunately the animals did not survive the operation a sufficient length of time to allow observations upon the urine and he was compelled to content himself with demonstrating that products of autolysis were present in the lobes shut off from the circulation. Doyon and Dufourt[15] report that upon tying off the hepatic artery they obtained a diminished formation of urea and increase in ammonia. Their results were somewhat unsatisfactory, however.

From the clinical side of the question quite recently a considerable amount of data which bears upon the question at hand has accumulated. Many investigators have studied the urinary changes occurring in certain metabolic disorders associated with hepatic diseases. Thus Schittenhelm[16] reports that in chronic diseases of the liver the ammonia output in relation to the total nitrogen elimination is increased. Axisa[17] states that in liver abscesses the same change is associated with a marked decrease in the urea percentage. Ingelrans and Dehons[18] corroborate these findings in hepatic insufficiency and claim that cirrhosis gives the same picture as acute yellow

[14] Jacoby, M., Ueber die fermentative Eiweissspaltung und Ammoniakbildung in der Leber, *Zeit. f. physiol. Chem.*, 1900, xxx, 149.

[15] Doyon, M. and Dufourt, L., Contribution à l'étude de la fonction ureopoietique du foie; Effets de la ligature de l'artère hepatique et de alle de la veine porto (*Arch. de physiol. normal et path.*, 1898, S. 5, x, 522), Ref. in *Maly's Jahresbericht f. Thierchemie*, 1898, xxviii, 382.

[16] Schittenhelm, A., Zur Frage der Ammoniakausscheidung im menschlichen Urin, *Deut. Arch. f. klin. Med.*, 1903, lxxvii, 517.

[17] Axisa, E., Ueber Harnstoff und Ammoniakausscheidung im Harn bei Leberabszess, *Zent. f. innere Med.*, 1905, xxvi, 929.

[18] Ingelrans, L., and Dehons, M., La valeur clinique de quelques signes urinaires considérés comme révélateurs de l'insuffisance hepatique, *Arch. de* med. exper. et d'anat. path., 1903, xv, 188.

atrophy. De Rossi,[19] on the other hand, offers evidence that not all diseases in which lesions of the liver are present show this altered relation of urea to ammonia elimination and concludes that the liver is not the only seat of the formation of urea.

In regard to acute yellow atrophy, vomiting of pregnancy, eclampsia, delayed chloroform poisoning and phophorus poisoning, recent investigations seem to show that the hepatic lesions, which are found to be present at autopsy in these conditions, are an important causative factor in the disturbance of metabolism. This disturbance shows itself in the urine by a marked diminution in the output of urea and an increase of the ammonia in relation to the total nitrogen elimination. Williams[20] assumes that the urinary picture of pernicious vomiting of pregnancy with its high percentage ammonia output is sufficiently definite to render it a valuable aid in determining the question of inducing labor. On the other hand. he contends that in the condition known as eclampsia there is a diminution in the total nitrogen and percentage urea with no very pronounced ammonia variation. Stone[21] believes that the vomiting of pregnancy is the result of a toxæmia, the lesions of which are primarily an acute degeneration of the liver amounting sometimes to necrosis and resembling in the fatal cases those of acute yellow atrophy. Zweifel's[22] researches confirm the opinion that the causative factor in eclampsia is a diminished oxidation which shows itself in the production and elimination of considerable quantities of p-lactic acid. The increased ammonia output is the result of the neutralization of the excess of acids produced and the diminished urea is due to the removal of quantities of ammonia which normally would be synthesized into urea.

[19] De Rossi, S., Sul valore semeiologico dell'urea et dell'ammonica nelle lesioni epatiche, *Riforma Medica*, 1904, xx, 1177.

[20] Williams, J. W., Pernicious Vomiting of Pregnancy, *Surgery, Gynecology and Obstetrics*, 1905, i, 41; *Johns Hopkins Hospital Bul.*, 1906, xvii, 71; *Amer. Jour. Med. Sciences*, 1906, cxxxii, 132.

[21] Stone, W. S., The Toxæmia of Pregnancy, *Amer. Gynecology*, 1903, iii, 518; Some Further Notes on the Toxæmia of Pregnancy, *Med. Record*, 1905, lxviii, 295.

[22] Zweifel, Zur Aufklärung der Eklampsie, *Arch. f. Gyn.*, 1905, lxxvi, 537.

Ewing and Wolf[23] report observations made upon pregnant women from the results of which they conclude that the various conditions of eclampsia, vomiting of pregnancy and yellow atrophy are but different degrees or manifestations of the same disordered process which probably centers itself in the hepatic cells and leads to the deranged elimination of urea and ammonia. A similar disturbance of metabolism associated with necrosis of the liver, has been found by Bevan and Favill[24] in fatal chloroform poisoning.

In view of all this it can readily be seen that the concensus of opinion favors the idea that the changes in the percentage elimination of urea and ammonia which are found to occur in these various conditions are but indications of the same functional lesion which centers itself in the hepatic cell. When necrosis of the liver occurs the cells which ordinarily synthesize ammonia into urea are out of function and the ammonia elimination is increased and the urea correspondingly falls.

With a full appreciation of the necessity of caution in transcribing deductions from the results of animal experiments to the explanation of pathological variations in the human organism, we feel that our results render somewhat doubtful the relationship between the hepatic necrosis of the vomiting of pregnancy, for example, and the urinary finding of a high percentage ammonia output. Wolf[25] has already justly criticized the conclusions of Williams in this regard and emphasizes the well-known fact that equally high percentages of ammonia are to be found when for any reason, as in inanition, the nitrogen or the calorific value of the diet becomes insufficient for the replacement of the wear and tear of the cell.[26] Schittenhelm has shown the influence of diet in this connection by experiments in chronic hepatic diseases where a high ammonia output is present. He noticed that upon increasing the fat of the diet

[23] Ewing, J. and Wolf, C. G. L., The Clinical Significance of the Urinary Nitrogen, II. The Metabolism in the Toxæmia of Pregnancy, *Amer. Jour. of Obstetrics*, 1907, lv, 289.

[24] Bevan, A. D., and Favill, H. B., Acid Intoxication and Late Poisonous Effects of Anæsthetics, *Jour. Amer. Med. Assoc.*, 1905, xlv, 691.

[25] Wolf, C. G. L., The Chemistry of Toxæmias in Pregnancy, *New York Med. Jour.*, 1906, lxxxiii, 813.

[26] Folin, O., Laws Governing the Chemical Composition of Urine, *Amer. Jour. of Physiol.*, 1905, xiii, 66.

a still further increase in the elimination of ammonia occurred and believes that the ammonia offers simply an indication of the lack of normal oxidation or catabolism of the ingested fatty acids. An examination of the results presented by Williams makes it seem very plausible that the diet in his cases is not an unimportant factor in the results. The figures for the total nitrogen indicate that the patients were practically in a state of diminished nutrition even approaching inanition since the amounts fall anywhere between four to eight grams per day, and more important still, the higher the total nitrogen the lower the ammonia and *vice versa* regardless of the severity of the condition. This same criticism can also be applied to the results of Ewing and Wolf. The daily total nitrogen elimination in their experiments is quite as low as that found by Williams, hence the high ammonia can be equally well attributed, in part at least, to similar causes.

The factor of low and insufficient diet was excluded in our experiments since the animals were upon exact equilibrium. We did obtain, however, severe hepatic lesions consisting of localized or diffuse necrotic areas and the ammonia output of our animals never showed more than the merest increase which was exceedingly transient. At this place emphasis must be laid upon the one experiment in which results comparable to, if not as pronounced as those of Williams and of Ewing and Wolf, were obtained. In this instance (see Table II.), however, the histological findings indicated that we were dealing not with a lesion of necrotic character but with an extensive and diffuse degeneration.

On account of the loose use of terms in pathology, this would seem to emphasize that a clear-cut differentiation between degeneration and necrosis[27] must be made histologically if we are to correlate the results of chemical studies and histological findings. The pathological condition "degeneration" does not imply autolysis which occurs only in necrosis. It is evident that one may occur without the other and therefore that the chemistry of the cell depends on its functional activity as determined by its physical state. As a reasonable explanation of why a difference in metabo-

[27] See first paper of this series, "The Hexon Bases," in this number of the *Journal.*

lism must be expected in the conditions of degeneration as opposed to necrosis we would present the following: In a generalized hepatic degeneration the lesion affects the protoplasm of each and every cell of the whole organ without destruction of the nucleus. This degeneration may set up enzymotic disturbances, secondary in character, which are not connected directly with the actual life processes of the cell and which may readily again return to normal when the abnormal conditions of the cell are removed. Such a differentiation between the actual life processes of the cell and those of a secondary functional character finds best expression in the German words "Baustoffwechsel" and "Betriebstoffwechsel." When the disturbance of enzymotic equilibrium occurs, if we grant for the sake of argument the unproven hypothesis that the urea formation is the result of enzymotic relations, there would take place an interference in the production of urea from ammonium compounds without an increase in the output of total nitrogen. A simple rearrangement in the partition factors would evidence itself according to which the ammonia would increase as the urea correspondingly diminished. This is exactly the condition found in our experiment with diffuse degeneration.

On the other hand, in necrosis, the individual cell is destroyed and all its functions cease. Autolysis begins in the same way as it does when death supervenes as the result of the removal of the cell from the body. Under such circumstances there occurs a true protoplasmic decomposition from which the cell can never recuperate. Here the nucleus becomes involved as is shown by the histological picture and in the urine by the occurrence of a marked increase in the elimination of uric acid, purin bases[28] and phosphorus.

In necrosis, moreover, although many individual cells are dead and have ceased to functionate, there always remain, unless the whole organ becomes necrotic, in contradistinction to the condition of degeneration, many normal cells, ready and capable of assuming in a vicarious manner the function of those already dead. This "factor of safety" in the liver is well demonstrated by the partial extirpation experiments of Ponfick, while the power of other organs

[28] For a detailed discussion of this subject see fourth paper of this series, "Nuclein Metabolism," in this number of the *Journal.*

to assume the urea-forming function is shown by the numerous Eck fistula experiments.

In necrosis, therefore, all that is expected as a urinary finding is the appearance of an increase in the total nitrogen output and of the abnormal products of autolysis such as proteoses, polypeptids and amino-acids; and even these latter, in scattered focal necrosis, need not necessarily appear since as very little liver tissue is destroyed the remaining normal cells still possess the power of splitting these substances, formed by cellular digestion, just as they do similar products of intestinal digestion brought to them by the portal[29] vein.

Our results substantiate this theoretical expectation. In the experiments with diffuse necrosis a marked and continued augmentation in the total nitrogen and urea elimination occurred as the result of the removal of the products of autolysis. The diminution in the ammonia output may be ascribed naturally to the increase in the proteid catabolism. Finally the increase in undetermined nitrogen is not definitely to be ascribed to the amino-acids ordinarily considered in this connection since we have not found leucin and tyrosin in the urine in amounts which would compare with those found in acute yellow atrophy.[30] It is to be considered rather as in the "colloidal" form as described by Salkowski and Mancini.[31]

SUMMARY.

1. In focal and diffuse necroses of the liver due to hæmotoxic sera there occurs an increased elimination of total nitrogen with a corresponding augmented output of urea. The ammonia excretion becomes slightly diminished at first, but later rises somewhat above normal. The undetermined nitrogen is markedly increased.

2. In diffuse degeneration with no necrosis on the other hand only a slightly increased output of total nitrogen is evident. A

[29] Freund, E. and Tœpfer, G., Ueber den Abbau des Nahrungseiweisses in der Leber, *Zeit. f. exp. Path.*, 1906, iii, 632.

[30] Riess, L., Phosphorvergiftung und Leberatrophie, *Berl. klin. Woch.*, 1905, xlii (Ewald Festnummer 44 a, 54).

[31] Mancini, S., Studi un nuovo segno per la diagnosi di insufficienza epatica; Contributo allo studio dell'azeta colloidale nelle urine normali e pathologiche, (*Arch. di farmocol. speriment,* 1906) Ref. in *Biochem. Cent.*, 1906, v, 549.

rearrangement of the urea-ammonia proportion occurs in that the ammonia excretion is augmented while the urea elimination is correspondingly diminished. The undetermined nitrogen rises but little.

3. In control experiments with normal serum no effect is produced.

4. These results would appear to indicate that in lesions characterized by uniform degeneration of the liver parenchyma, in contradistinction to necrosis, there occurs no increased nitrogen elimination but merely a disturbance of the urea-forming function of the cell without the appearance in the urine of products of autolysis. On the other hand in necrosis, of even considerable extent, the total-nitrogen is greatly augmented, as is also the rest-nitrogen; while the production of urea, on account of the persistence of normally functioning liver cells, remains relatively unchanged.

This " factor of safety "[32] possessed by the liver is, we think, one of the most important results brought out in this investigation and must be given great weight in any consideration of the chemistry of hepatic disturbances.

[32] Meltzer, S. J., The Factors of Safety in Animal Structure and Animal Economy, *Jour. of Amer. Med. Assoc.*, 1907, xlviii, 655.

EXPERIMENTAL LIVER NECROSIS; IV. NUCLEIN METABOLISM.[1]

By HOLMES C. JACKSON, Ph.D.,
Adjunct Professor of Physiological Chemistry,

AND

RICHARD M. PEARCE, M.D.,
Professor of Pathology and Bacteriology,
Albany Medical College.

(*From the Bender Laboratory, Albany, N. Y.*)

The experiments here detailed were undertaken for the purpose of determining the process or processes intimately concerned in the increased elimination of uric acid which, as has been shown elsewhere,[2] accompanies the augmented output of total nitrogen when hæmotoxic serum is injected. The fact that the principal lesion produced by the toxic serum is in the liver lends peculiar interest to the problem, in view of the important part which this organ is supposed to play in the formation of endogenous uric acid. The direct object of the experiments was to determine whether the increased elimination of uric acid in dogs under these conditions was the result of the breaking down of nuclear material during the necrosis which follows the injection, or whether it signified simply a diminished oxidative power on the part of the hepatic cell by which the uric acid normally oxidized to simple complexes is eliminated unchanged. Or, more concisely, does an actual increase in the production of uric acid from the nucleic acids of the decomposed nuclei of the necrotic cell occur or is there a simple rearrangement of enzymotic equilibrium by which less uric acid is decomposed than normally.

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Received for publication July 2, 1907.

[2] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

Schittenhelm[3] has isolated two enzymes from the spleen and liver, one of which causes a hydrolytic splitting off of the amino group of guanin and adenin transforming these bodies thereby into xanthin[3] and hypoxanthin respectively; the other an oxydase, xantho-oxidase (Jones), oxidizes the latter compounds into uric acid. Since adenin, guanin, xanthin and hypoxanthin are found in varying amounts in the different nucleic acids which give character to the nucleoproteid of the nucleus we have a fairly definite process by which uric acid results, it may be assumed, from the decomposition of the nucleus by means of autolytic enzymes aided perhaps by a special oxydase.

On the other hand, however, the fact has long been known that various tissues are capable of decomposing uric acid. Wiener,[4] Schittenhelm[5] and Almagia[6] have recently studied the question in detail and describe the presence of uricolytic enzymes in various tissues of several animals. Schittenhelm claims that the calf's kidney is most active in this regard with the liver of the same animal second in power. Pfeiffer[7] was able to prove that ninety to ninety-five per cent. of the uric acid added to human kidneys suffered decomposition. In the horse Almagia found the most active enzyme to be in the liver. These results indicate that the liver among other organs possesses the power of forming uric acid by oxidization and perhaps also by synthesis,[8] while at the same time it is

[3] Schittenhelm, A., Ueber die Harnsäurebildung in Gewebsauszügen, *Zeit. f. physiol. Chem.*, 1904, xlii, 251; Ueber die Harnsäurebildung und die Harnsäurezersetzung in dem Auszügen der Rinderorgane, *ibid.*, 1905, xlv, 121.

[4] Wiener, H., Ueber Zersetzung und Bildung der Harnsäure im Thierkörper, *Arch. f. exper. Path. u. Pharm.*, 1899, xlii, 375.

[5] Schittenhelm, A., Ueber das uricolytische Ferment, *Zeit. f. physiol. Chem.*, 1905, xlv, 161.

[6] Almagia, M., Zur Lehre vom Harnsäurestoffwechsel, Ueber die Zersetzung der Harnsäure durch die Organe des Saugetheirs, *Beit. z. chem. Phys. u. Path.*, 1905, vii, 459.

[7] Pfeiffer, W., Zur Lehre vom Harnsäurestoffwechsel, Ueber die Zersetzung der Harnsäure durch menschliche Nierengewebe, *Beiträge z. chem. Phys. u Path.*, 1905, vii, 463.

[8] Burian, R., Ueber die oxydative und die vermeintliche synthetische Bildung von Harnsäure im Rinderleber Auszug, *Zeit. f. physiol. Chem.*, 1905, xliii, 497; Die Herkunft der endogenen Harnpurine bei Mensch und Saugethier, *ibid*, 1905, xliii, 532.

capable of decomposing it through the stages of allantoïn and glyoxylic acid.

Austin[9] has raised the point, however, that the alkali in which the uric acid is dissolved is capable of splitting up the latter and that all preparations of uricolytic enzymes contain purin bases which readily become transformed into uric acid. Notwithstanding the latter criticism it seems fairly well founded that the reaction under discussion is a reversible one in which synthetic and analytic processes come into a state of equilibrium in the cell and that alterations in the activity of one or the other set of enzymes increase or diminish the elimination of uric acid without the occurrence, necessarily, of an increase or decrease in the amount of purin material formed from the nucleic acid of the nucleus.

If the increased elimination of uric acid in dogs injected with toxic sera is the result of altered uric acid equilibrium of the cell, as just described, one would expect to find that, as more xanthin the hypoxanthin are oxidized to uric acid, less of these purins should be eliminated in the urine. On the other hand, if the uric acid is the result of a new formation from the nucleic acids then an increase in purins also would be expected and at the same time an augmentation in elimination of phosphoric acid in some form[10] as the result of the splitting of the nucleic acid molecule. In the formation of uric acid by the first process no change in phosphoric metabolism should take place.

Methods.—The experiments presented in Tables I, II and III were carried out in the following manner: The animals were placed upon nitrogenous equilibrium after which estimations were made during a three day fore period. An injection was then given, and in two instances a second injection after a lapse of one and three days respectively. The observations were continued until after the maximum effect was reached. The purin-free diet was a casein,

[9] Austin, A. E., The Uricolytic Enzyme in Animal Organs, *Jour. of Med. Research,* 1906, xv, 309. The Uricolytic Enzyme, *ibid.*, 1907, xvi, 7.

[10] Jackson, H. C. and Blackfan, K. D., Action of Certain Drugs on the Elimination of Uric Acid During a Nitrogen-free Diet, *Albany Medical Annals,* 1907, xviii, 24.

cracker dust and lard mixture. The urine was examined for total-nitrogen by the Kjeldahl-Gunning method, the uric acid and purin bases were determined by the Salkowski procedure, and the inorganic phosphates by titration with uranium nitrate, using potassium ferrocyanide as an indicator. The method used for total phosphorus was fusion of the evaporated residue of the urine with sodium hydroxide and potassium nitrate, precipitation with ammonium molybdate in the presence of ammonium nitrate, solution of the ammonio-phosphomolybdate in ammonia and re-precipitation with magnesium mixture. This precipitate was filtered off, incinerated and weighed as magnesium pyrophosphate.

The question of the presence of phosphorus in the urine in organic form has recently received attention from various investigators among whom Bergmann[11] and LeClerc and Cook[12] incline to the opinion that all of the phosphorus of the urine exists in the inorganic form. The latter observers studied the phosphorus content of the urine by means of determinations made by Neumann's[13] method, and by that outlined above, and compared the results with those obtained by the uranium-titration procedure. They show that the results by the dry fusion method are uniformly higher by three to four per cent. than those given either by uranium acetate or by Neumann's method; hence they incline to the view that this difference is within the limits of error and that no organic phosphorus is present in normal urine.

We cannot agree with these conclusions in view of the results obtained by one of us in a study of the elimination of organic phosphorus after the administration of sodium salicylate.[14] Nor do they receive support from the results which are presented in this

[11] Bergmann, W., Ueber die Ausscheidung der Phosphorsäure beim Fleisch- und Planzenfresser, *Arch. f. exper. Path. u. Pharm.*, 1901, xlvii, 77.

[12] LeClerc, J. A., and Cook, F. C., Metabolism Experiments with Organic and Inorganic Phosphorus, *Jour. of Biol. Chem.*, 1906, ii, 203.

[13] Neumann, A., Einfache Veraschungsmethode (Säuregemisch-Veraschung), *Zeit. f. physiol. Chem.*, 1902, xxxvii, 115.

[14] Jackson, H. C. and Blackfan, K. D., Action of Certain Drugs on the Elimination of Uric Acid During a Nitrogen-free Diet, *Albany Medical Annals*, 1907, xviii, 24.

communication. The constant uniformity of difference shown by the fusion method over that of titration seems to indicate that the difference is not due to the factor of error in the method. We have performed in this connection some preliminary experiments with a view to explaining the differences reported and expect to continue them more in detail. At present we can say that Neumann's method apparently yields results which agree closely with those obtained by titration with uranium nitrate, using potassium ferrocyanide as indicator. When the fusion method is employed the figures obtained are uniformly higher than by both of the other methods. At present, therefore, we incline to the opinion that phosphorus in other than inorganic form is present in normal urine.

TABLE I.

Nuclein Metabolism. Dog 59.

Date.	Grams Nitrogen as			Grams P_2O_5.		Notes.
	Total.	Uric Acid.	Purin Bases.	Inorganic.	Organic.	
Apr. 10	7.86	0.0015	0.001	0.899	0.147	
11	5.51	0.0159	Lost	0.652	0.093	
12	4.70	0.0141	0.0082	0.690	0.087	
13	4.49	0.0121	0.0097	0.576	0.050	Injection 10 A. M. toxic serum; dose 1:785.
14	6.97	0.0126	0.0231	1.009	0.085	
15	12.50	0.0246	0.0169	0.690	0.121	Injection 11 A. M. toxic serum; dose 1:1745.
16	11.08	0.0127	Lost	0.848	0.072	Hburia.
17	13.81	0.0319	0.0287	0.629	0.083	
18	11.93	0.0203	0.0265	0.866	0.023	Killed.

TABLE II.

Nuclein Metabolism. Dog 61.

Date.	Grams Nitrogen as			Grams P_2O_5.		Notes.
	Total.	Uric Acid.	Purin Bases.	Inorganic.	Organic.	
Apr. 22	3.33	0.0042	0.0086	0.478	0.078	
23	3.83	0.0063	0.0072	0.554	0.052	
24	3.12	0.0050	0.0079	0.584	0.097	Injected 12 M. toxic serum; dose 1:937; vomited.
25	4.57	0.0167	0.0103	0.549	0.085	Hburia.
26	5.74	0.0336	0.0107	0.387	0.074	Hburia.
27	6.83	0.0241	0.0124	0.387	0.084	
28	6.54	0.0200	0.0235	0.326	0.046	
29	5.69	0.0112	0.0086	0.356	0.118	Killed.

TABLE III.

Nuclein Metabolism. Dog. 63.

Date.	Grams Nitrogen as			Grams P_2O_5.		Notes.
	Total.	Uric Acid.	Purin Bases.	Inorganic.	Organic.	
May 14	4.74	0.0057	0.0061	0.520	0.062	
15	4.41	0.0054	0.0068	0.557	0.109	
16	4.26	0.0068	0.0076	0.550	0.072	Injected 11 A. M. toxic serum; dose 1 : 835; vomited.
17	5.29	0.0249	0.0110	0.641	0.062	
18	4.24	0.0143	0.0088	0.508	0.078	
19	4.34	0.0109	Lost.	0.535	0.066	Injected 11 A. M. toxic serum; dose 1 : 557; vomited.
20	4.75	0.0133	0.0181	0.576	0.066	Hburia.
21	7.64	0.0141	0.0116	0.435	0.090	Hburia.
22	8.38	0.0095	0.0187	0.440	0.075	Hburia.
23	6.21	Lost.	0.0133	0.550	0.111	Killed.

Results.—From the figures in the above tables it is evident that the increase of uric acid after injection, as noted in the study of the nitrogenous metabolism,[15] is constant and that there also occurs an increase of purin bases and with one exception of phosphorus pentoxide. This general increase occurs not only after the first but also after subsequent injections. It reaches its maximum on the second day and then falls away. The uric acid increase is greater proportionately than that of the total nitrogen, hence an augmentation of the percentage uric acid in terms of total-nitrogen takes place. The uric acid nitrogen, however, returns to the normal sooner than the latter. The same facts hold true for the purin bases the elimination of which runs parallel, in a general way, to the uric acid output. In the one instance (Table II, Dog 61) in which the phosphorus pentoxide elimination remained constant there still occurred the markedly increased uric acid and purin base output. That the phosphorus pentoxide elimination did not follow that of the uric acid and purin bases is to be explained by the fact that after the injection the animal ate only a quarter of the daily food allowance. Although on the day following the injection the

[15] See third paper of this series "Nitrogenous Metabolism" in this number of the *Journal.*

amount ingested of phosphorus pentoxide was therefore diminished, the elimination of inorganic phosphates remained unchanged. This would indicate that an actual increase in endogenous phosphorus pentoxide formation took place, thus bringing the apparent exception in agreement with our other observations. The organic phosphorus elimination was unaltered.

The question as to the cause of the increased output of uric acid during a purin-free diet, observed after various experimental procedures, has been variously explained. Beebe[16] concludes that the increase found after alcohol administration is to be attributed to a diminished oxidative power on the part of the hepatic cells although the purin bases were likewise increased. In commenting upon this view Jackson and Blackfan, from results which they obtained with alcohol, inclined to the opinion that the facts would warrant rather the assumption that the alcohol causes an increased new formation of uric acid and purin bases, and they adduced as evidence, among other factors, the augmented excretion of organic phosphorus.

According to the view of Schittenhelm, concerning the probable way in which the uric acid is formed from the nucleo-proteid, we would expect, if for any cause oxidization in the hepatic cell is diminished, the xantho-oxydase would be affected, and as a result less uric acid would be formed from the purin bases than under normal circumstances. An increase in purins would also occur if the hydrolytic splitting enzymes were unaffected. An augmentation of uric acid would take place either as the result of a diminished power of the uricolytic enzymes or as the result of an increased new production, other things being equal. In the latter case the purin would also be increased.

The results reported in the paper on nitrogenous metabolism do not indicate that the injection of a hæmotoxic serum causes a prolonged or decided decrease in the oxidative power of the hepatic cell. As a whole the organ continues to perform its functions in the normal way. We must therefore explain the increase in purins and uric acid as a decomposition of nuclear material in the autolysis occurring in the areas of necrosis. This is brought about by

[16] Beebe, S. P., The Effect of Alcohol and Alcoholic Fluids upon the Excretion of Uric Acid in Man, *Amer. Jour. of Physiol.*, 1904, xii, 13.

the hydrolysis of the nucleic acids. Such an explanation agrees with the feeding experiments performed by Sweet and Levene[17] upon a dog with an Eck fistula. They found the ingestion of nucleic acids to be followed by a rise in uric acid and total phosphorus elimination. The fact that the increased excretion of these nuclear compounds usually reaches its maximum in our experiments on the second day, when the autolysis is at its height, would also strengthen this explanation.

The increased elimination of inorganic phosphates which accompanies the output of uric acid would likewise point to the new formation of the latter from the nucleic acids formed as a step in the autolysis of nuclear material. The phosphoric acid radical of these acids is evidently excreted in inorganic form and thus the mechanism differs from that observed in connection with the administration of alcohol and salicylic acid under which circumstances the phosphoric acid is apparently eliminated in organic combination, since no increase in inorganic phosphates occurs.

Allantoïn.—In the dog, the largest part of the quantity of uric acid ingested, as shown by Swain,[18] disappears and is not excreted. In the experiments outlined above it is not improbable, therefore, that a much larger amount of uric acid was produced in the necrosis of the cell nucleus than was eliminated. Of the total amount formed only a small proportion escapes hydrolysis and appears in the urine. Swain also has shown that the ingestion of large amounts of uric acid is followed by the elimination of small quantities of allantoïn.

In order to obtain evidence upon this point the urine of the six animals under observation in the experiments reported in the paper on nitrogenous metabolism, was examined for allantoïn according to the method of Loewi.[19] The results are not uniform but indicate that usually the increase of uric acid is accompanied by an increase in allantoïn; for example, in one the amount of uric acid the day

[17] Sweet, J. E., and Levene, P. A., Nuclein Metabolism in a Dog with Eck's Fistula, *Jour. of Exper. Med.*, 1907, ix, 229.

[18] Swain, R. E., Formation of Allantoïn from Uric Acid, *Amer. Jour. of Physiol.*, 1901, vi, 38.

[19] Loewi, O., Beiträge zur Kenntnis des Nucleinstoffwechsels, *Arch. f. exp. Path. u. Pharm.*, 1900, xliv, 20.

before injection was 0.018 gram and of allantoïn 0.319; in the urine of the twenty-four hour period following the former rose to 0.337 and the latter to 0.669.

CONCLUSIONS.

In necrosis of the liver of the dog produced by hæmotoxic immune sera, the increased excretion of uric acid, purin bases and inorganic phosphorus pentoxide is the result of the hydrolysis of nuclear material occurring during the autolysis of the necrotic tissue.

EXPERIMENTAL LIVER NECROSIS. V. THE FATS AND LIPOIDS.[1]

By HOLMES C. JACKSON, Ph.D.,
Adjunct Professor of Physiological Chemistry,

AND

RICHARD M. PEARCE, M.D.,
Professor of Pathology and Bacteriology,
Albany Medical College.

(*From the Bender Laboratory, Albany, N. Y.*)

The results outlined in this communication constitute a partial report of a somewhat comprehensive investigation[2] now in progress of the chemical processes concerned in the variations occurring in the amounts of fats and lipoids in the hepatic cell under normal and various pathological conditions.

It has seemed advisable, in connection with the other investigations of liver necrosis here presented, to discuss at this time only that part of the general study which deals with the fatty changes in hepatic necrosis brought about by the injection of hæmotoxic immune serum.

The study of such lesions is of peculiar value in view of the attempts which have been made to bring into a relation of cause and effect the autolysis of the organ and the appearance of the fat. We have therefore with this point in view attempted to determine whether in the necrosis (autolysis) which follows the injection of the serum there occurs any alteration in the fat content correspond-

[1] Conducted under grants from the Rockefeller Institute for Medical Research. Received for publication July 11, 1907.

[2] This investigation, including a study of the fatty changes occurring in various experimental lesions of the liver of the dog and of certain pathological conditions of the human liver, will be published later in full by H. C. Jackson and L. K. Baldauf. We wish to express here our indebtedness to Dr. Baldauf for the privilege of utilizing in this partial report that portion of his work which refers to necroses of the liver produced by hæmotoxic serum.

ing to changes observed in the nitrogenous constituents of the cell. Especial interest is attached to this question in view of the somewhat widely divergent opinions held as to the origin of the fat which appears in the so-called fatty transformation of various organs. That the fat does not arise from a peculiar decomposition of the proteid molecule in the cell, the fatty degeneration of Virchow, seems fairly well established. On the other hand, Rosenfeld[3] and others hold that the fat makes its appearance as a simple infiltration from without when for any reason the cell has received an injury which seems to inhibit its oxidizing power. The appearance of fat in organs during phlorhizin poisoning is thus explained by Lusk.[4] Waldvogel,[5] however, who has investigated this question most thoroughly believes that the process is one closely allied to autolysis. His theory is that normally certain substances, which may be called combined fats, such as the ovovitellin of Hoppe-Seyler, or the lecithalbumin of Liebermann, hold the fatty radical in a combination which does not react to microchemical fat stains, such as Scharlach R or Sudan III, and which cannot be removed chemically by the ordinary fat solvents. These substances, however, during autolysis become split in such a manner that the fat radical is liberated in the form of protagon, jecorin, lecithin and even neutral fats. He supports this contention by experiments upon the livers of phosphorus-poisoned animals and upon normal livers undergoing autolysis, in which he has shown there is a marked augmentation during autolysis of such substances as protagon, jecorin, cholesterin, fatty acids and neutral fats. The lecithin, on the other hand, is diminished.

[3] Rosenfeld, G., Fetbildung, *Ergebnisse der Physiologie*, 1902, i, 651; *ibid.*, 1903, ii, 50.

[4] Ray, W. E., McDermott, T. S. and Lusk, G., On Metabolism During a Combination of Phosphorus Poisoning and Phlorhizin Diabetes, *Amer. Jour. of Physiol.*, 1900, iii, 139.

[5] Waldvogel, Autolyse und fettige Degeneration, *Virchow's Arch.*, 1904, clxxvii, 1. Phosphorvergiftung und Autolyse, *Deut. Arch. f. klin. Med.*, 1905, lxxxii, 437. Waldvogel and Mette, Die Autolyse in Menschlichen fettige degenerierten Organen, *Münch. med. Woch.*, 1906, ix, 402. Waldvogel, Die durch Fermente bewirkten Umwandlungen bei der fettigen Degeneration, *Zeit. f. physiol. Chem.*, 1904, xlii, 200.

Siegert[6] has also shown that, although in autolysis the ether extract of the liver does not increase, a marked rearrangement of the fatty compounds of the extract takes place, and that the jecorin rapidly suffers decomposition. This is not in accord with Waldvogel's results. In this connection it may be mentioned that Taylor[7] has conducted experiments upon normal and phosphorus-poisoned frogs and finds that, although the absolute amount of free fat may not increase after the administration of phosphorus the combined fats estimated after digestion with pepsin-hydrochloric-acid suffer a marked diminution equivalent to two thirds of the amount originally present.

As stated in previous papers in this series phosphorus poisoning does not seem to set up in the liver processes which are strictly analogous to autolysis following necrosis, hence the results to be presented must not be considered as strictly comparable to those obtained in other lesions.

Few attempts have been made to study the effect of a true necrosis upon the fat constituents of the cell. Dietrich,[8] as the result of histological studies, claims that autolysis is not an important factor, since tissues introduced into the peritoneal cavity in collodion sacs do not show fatty change. When the tissue, however, is not enclosed in sacs, fat droplets appear which are present not in the cell substance but in the interstices of the tissue. He also tied off the renal arteries and found a "deposition" of fat around the necrotic areas. He believes, therefore, that fat will not appear if the cell is completely dead as in necrosis; but only when it continues to functionate incompletely, as, for example, must be the case with the cells around necrotic areas.

This opinion we can confirm as the result of the histological study of focal necroses caused by hæmotoxic serum. Frozen sections of formalin-hardened material stained with Scharlach R never show more fat, and usually less, than the surrounding adjacent

[6] Siegert, F., Das Verhalten des Fettes bei der Autolyse der Leber, *Beiträge zur chem. Physiol. u. Path.*, 1902, i, 114.

[7] Taylor, A. E., On Fatty Degeneration, *Jour. of Med. Research*, 1903, ix, 59.

[8] Dietrich, A., Experimente zur Frage der fettigen Degeneration *Münch. med. Woch.*, 1904, li, 1510.

normal liver tissue. There is always present, however, in lesions twenty-four to forty-eight hours old a very definite and striking accumulation of fat in the ring of more or less degenerated cells lying between the necrotic and normal liver. These cells correspond to those which in hæmatoxylin and eosin preparations present a vacuolated, granular protoplasm and pycnotic, poorly staining, nuclei.[9]

Di Cristina,[10] who conducted experiments somewhat after the nature of those of Dietrich, and made chemical analyses by Rosenfeld's method, states that no increase of fat occurs in the necrosis caused by shutting off completely the renal circulation. Of considerable interest in connection with Dietrich's view are the experiments reported by Bainbridge and Leathes.[11] These investigators ligated the hepatic artery alone and thereby obtained an increase in fat but no necrosis. The ligation of the portal vein on the other hand resulted in atrophy of the cells and some necrosis, but no augmentation of fat. These experiments appear to confirm the idea that the cells must retain in part their normal function and be normally bathed with the circulating fluids in order to give rise to the appearance of fat within them.

In concluding this brief discussion which merely suffices to indicate the trend of opinion in regard to the subject under investigation it may be said that much of the discrepancy in the results reported can be safely ascribed to the varying methods employed especially in connection with the extraction of the fatty material from the tissue. Siegert has also emphasized the ease with which the extracted products undergo laboratory changes.

Methods.—The organs were removed from the body quickly, put through a hashing machine, weighed, and dried under absolute alcohol at about 70–80° C. At this stage the partially dry material was weighed and then ground in a machine to an impalpable powder. Part of this was further dried in a desiccator to constant

[9] Pearce, R. M., Regenerative Changes in the Liver: A Study of Experimental Lesions in the Dog, *Jour. of Med. Research*, 1906, xv, 99.

[10] Di Cristina, Die chemischen Veränderungen bei fer fettigen Degeneration in Beziehung zur den anatomischen, *Virchow's Arch.*, 1905, clxxxi, 509.

[11] Bainbridge, F. A. and Leathes, J. B., The Effect of Arterial or Venous Obstruction upon the Nutrition of the Liver Cells, *Biochem. Jour.*, 1906, ii, 25.

weight and upon this was calculated the dry substance and nitrogen content of the original tissue. Another weighed part was extracted in a Soxhlet with alcohol and chloroform successively according to the method of Rosenfeld. Each total extraction with alcohol and with chloroform lasted on an average thirty hours. In some cases the original partly-dried material was so fatty that a rough extraction with chloroform at room temperature preceded the grinding. This extract was added to the subsequent one obtained from the Soxhlet. The total fat was taken up in a definite volume of chloroform. An aliquot portion of this was evaporated to complete dryness at about 70° C. and from this was calculated the fat per cent. of the tissue.[12]

For our purposes it was not thought necessary to keep separate for analysis the alcohol and chloroform extracts as Waldvogel does with alcohol and ether. This procedure is exceedingly time consuming and we have employed another and simpler method which we believe has given results equally definite. Instead of attempting to decide whether the fat compounds present in the extract underwent any change or rearrangement, such as is described by Waldvogel during autolysis of normal tissues and in phosphorus poisoning, it seemed sufficient to determine the nitrogen and phosphorus pentoxide content of the extract, and from these figures to calculate the relationship of the nitrogen to the phosphorus. Since the molecule of the lecithins contains one nitrogen and one phosphorus atom the relationship P : N = 1 : 1; in jecorin, however, this ratio is 1 : 4 and in protagon about the same, varying from 1 : 3.4 to 4.8. The latter figure is calculated from the analyses of Dunham.[13] It is seen, therefore, that the greater the preponderance of substances of the jecorin and protagon type in the extract the higher would be the P : N ratio. On the other hand, if these substances should undergo an autolytic change whereby lecithin and fatty acids were

[12] We have employed chloroform in this connection, since, in the first place, it is a much readier solvent for the fatty compounds than either sulphuric or petroleum ether, and secondly, because all of the ether we could obtain reacted distinctly acid to phenolphthalein, a fact already alluded to by Baldauf, Chemistry of Atheroma and Calcification (Aorta), *Jour. of Med. Research*, 1906, xv, 355.

[13] Dunham, E. K., Further Observations on the Phosphorized Fats in Extracts of the Kidney, *Proc. of Soc. for Exper. Biol. and Med.*, 1905, ii, 63.

produced, this ratio should fall to the neighborhood of 1 : 1. Hence from variations in this ratio, changes in the fatty constituents of the extract should be readily determined.

Measured portions of the chloroform extract were analyzed in duplicate as follows:[14]

Total nitrogen by the Kjeldahl-Gunning method; phosphorus pentoxide by the usual fusion procedure and weighing as magnesium pyrophosphate and the iodine equivalent as outlined by the Association of Official Agricultural Chemists.[15] With some exceptions portions of the tissues analyzed were stained with hæmotoxylin and with Scharlach R for the purpose of determining roughly in a comparative way the extent of the necrotic lesion and the fat content.

Results.—The table presents the results obtained in the analysis of four dog livers with normal, four to five, per cent. fat content, one apparently normal but very fatty liver with 21.9 per cent., five with focal necrosis and three with diffuse necrosis of varying degree. As can be seen no relation exists between the degree of necrosis and the amount of fat present. The high amount of fatty material which is present in the normal liver, extractable by the newer method of Rosenfeld, is at first glance surprising, but is in accord with the results of recent investigators. The fat per cent. of all the necrotic livers falls between the normal limits with the exception of 43 and 21 which are above the normal, but these do not represent the most extensive necrosis. Experiments 28 and 29, with the most diffuse necrosis, show normal amounts of fat.

The point which was emphasized in one of the previous papers[16] concerning the percentage of dry substance in the fatty livers is well shown in the table. Whenever the fat per cent. rose above normal the per cent. of dry substance rose almost proportionately. This relation is readily seen by referring to that column in the table in which is given the per cent. of fat-free dry substance. With two exceptions, the dry substance without fat falls between 18.8 to

[14] In most instances the chloroform had to be *completely* removed before the commencement of the analysis.

[15] U. S. Dept. of Agriculture, 1899, Bulletin 46, 50.

[16] See first paper of this series, "The Hexon Bases" in this number of the *Journal.*

TABLE I.

Fats and Lipoids.

Experiment.	Tissue						Alcohol-chloroform Extract.				Fat by Scharlach R.	Lesion.
	Per Cent.						Per Cent.		P:N	Iodine Equivalent.		
	Dry Substance	Dry Substance. Fat-free.	Fat.		Nitrogen.		Nitrogen.	P_2O_5.				
			Moist Substance.	Dry Substance.	Dry Substance.	Dry Substance. Fat-free.						
1	25.6	22.6	3.97	15.5	12.7	15.0	—	—	—	58.3	+	Normal.
5	28.6	24.1	4.55	15.9	9.7	11.5	1.8	2.6	1:7.0	60.0	?	Normal.
2	24.5	19.5	4.95	20.2	12.7	15.9	2.5	5.2	1:4.9	56.6	+	Normal.
4	18.5	13.7	4.75	25.7	9.2	11.3	1.5	3.6	1:4.4	53.4	+	Normal.
18	40.7	18.8	21.85	53.7	7.5	12.6	0.6	1.3	1:4.5	47.0	+++	Normal (very fatty).
7	29.8	26.7	3.10	10.4	9.3	13.2	3.1	7.3	1:4.3	67.7	?	Focal necroses.
6	25.3	21.2	4.12	16.3	12.5	16.7	2.4	7.0	1:3.5	58.3	?	Focal necroses.
26	28.3	24.5	3.84	13.6	9.9	13.8	2.1	5.0	1:4.3	58.7	+	Extentive focal necroses.
43	41.1	24.9	16.23	39.5	8.3	14.0	0.7	2.0	1:3.8	47.0	++	Extensive focal necroses.
27	26.0	20.3	5.72	22.0	11.5	15.5	1.5	6.5	1:2.4	49.3	++	Extensive focal necroses.
21	41.1	14.9	25.15	61.2	6.7	10.5	0.3	1.1	1:3.0	50.0	+++	Diffuse necrosis.
28	23.2	18.8	4.43	19.1	11.9	15.5	2.3	5.8	1:4.3	50.3	+	Diffuse necrosis.
29	21.9	20.1	4.88	19.5	12.7	16.3	2.3	6.1	1:4.0	62.3	+	Diffuse necrosis (most marked)

26.7 per cent. and this surprisingly small variation is in no definite relation to the amount of fat present. The two exceptions are one with normal and one with high fat content. Waldvogel has claimed that as the fatty autolysis increases the water content of the tissue also rises. We would be inclined to ascribe this rather to the nitrogenous autolysis *in vivo* which as we have pointed out elsewhere[16] tends to diminish the amount of dry substance if the circulation is not too greatly impaired. This is shown clearly in Experiments 27, 21, 28 and 29, with the most pronounced necrosis, in which it is seen that the figures for the dry fat-free substance lie on the lower edge of the variation limit for this factor.

As regards the nitrogen content of the tissues a somewhat similar condition of affairs is evident, and although this point does not come directly into this part of the general subject it is of considerable interest. It will be seen that the per cent. nitrogen of the fat-containing dry substance varies within rather wide limits (6.7 to 12.7 per cent.). In those instances, however, where the fat content is high the nitrogen per cent. is low, as would be expected. If, however, the nitrogen of the fat-free dry substance is considered, it is found that the figures for this factor are surprisingly constant throughout (10.5 to 16.7 per cent.). Some of the lowest and highest values occur where, as regards the fat content, the liver tissue was perfectly normal. This indicates apparently that in conditions such as those under discussion, when an increase in fat-content occurs, the material which is represented by the nitrogen of the tissues suffers no decrease or increase in amount. If then the fat originates from some compound proteid antecedent, the proteid component remains apparently unchanged.

A consideration of the character of the fatty extract obtained from normal and necrotic tissues presents some interesting facts. In regard to the iodine equivalent, which indicates roughly the content of oleic acid in the fat mixture, it is evident that as the fat per cent. increases the iodine absorption factor falls below normal (Experiments 18, 43, 27 and 21). This low iodine factor associated with increased fat content would of course point to a diminishing content of oleic acid radicals as the fat is heaped up in the cell. This again is directly opposed to the finding of Waldvogel in phosphorus poisoning.

An examination of the nitrogen and phosphorus pentoxide percentage of the fatty extracts indicates that somewhat wide variations are present, the nitrogen varying from 0.3 to 3.1 and the phosphorus pentoxide from 1.1 to 7.3. It is evident, however, that the lower percentages are in those experiments in which the greatest amount of fat is present (Experiments 18, 43, 27 and 21). With these exceptions the percentage figures are quite regular. Only one explanation can be made of this difference. That is, that as fatty compounds make their appearance in the cell, those substances predominate which do not contain nitrogen or phosphorus, namely the neutral fats, fatty acids and cholesterin.[17]

Of particular interest is the ratio of phosphorus to nitrogen discussed above. It is seen from an examination of the figures in the table that this ratio remains remarkably constant regardless of the absolute variation in the figures. The ratio, with the exception of Experiment 5 which is high, varies only from 1 : 2.4 to 4.9. If we also exclude Experiments 21 and 27 the variation is still further reduced to 1 : 3.5 to 4.9, which is well within the limits of error or better perhaps within the calculation error as made from the published figures for the nitrogen and phosphorus percentages of such poorly defined substances as protagon and jecorin. This high ratio indicates that the nitrogen- and phosphorus-containing fats present in the extracts are of the protagon and jecorin type and remain so irrespective of the amount of fat appearing in the cell.

It must be admitted that some slight evidence does exist to indicate that perhaps under certain conditions these bodies may undergo autolysis. In the two experiments, for example 21 and 27 with a low ratio (1 : 2.4 to 3.0), considerable autolysis must have been going on during the extensive necrosis and the low ratio as a concomitant factor would point to the appearance in the extract of bodies such as the lecithins, which as previously stated possess a ratio of 1 : 1. In these experiments roughly one third of the nitrogen- and phosphorus-containing fats has been replaced by lecithin, according to this reasoning. We are somewhat sceptical, however, of the validity of this line of argument since it does not

[17] We possess confirmatory evidence upon this point in the results which have been obtained with the saponification equivalent. These will be published in a later paper.

agree with Waldvogel's observations; nor is the low ratio present in the two experiments (28 and 29) where one would expect it to be most markedly diminished since the necrosis was most pronounced. In these two instances, however, the ratio is normal. As the low ratio occurs in only two out of the eight experiments in which necrosis was present, we are more inclined to believe that some other factor is the true cause for the change.

SUMMARY.

1. Changes which occur in the fat content of the liver of dogs receiving hæmotoxic serum bear no relation to the degree of necrosis produced by this serum.

2. An increase in water content of the tissue seldom occurs, but where present is due to the nitrogenous autolysis rather than to the deposition of fat.

3. The appearance of fat in the cell is not associated with a decomposition of the proteid component of the compound fats, but rather to a simple splitting off of the fatty radical. This is shown by the slight variations occurring in the percentage nitrogen of the fat-free substance.

4. The iodine equivalent diminishes as the fat content increases. This would indicate that in the fatty changes which occur, fats other than those containing oleate radicals make their appearance.

5. The ratio of phosphorus to nitrogen in the alcohol-chloroform extract remains practically constant in all degrees of necrosis. Hence the substances of the protagon and jecorin type hold the same relation to the lecithins during the autolysis as they do normally.

6. In a general way it may be said that the results obtained in the microchemical staining of the fats with Scharlach R agree with those found by chemical extraction methods.

BIOLOGICAL RELATIONSHIPS OF DIPLOCOCCUS INTRACELLULARIS AND GONOCOCCUS.[1]

By MARTHA WOLLSTEIN, M.D.

(*From the Rockefeller Institute for Medical Research, New York.*)

Recent studies by Brickner and Cristéanu[2] having shown a marked similarity between *Diplococcus intracellularis* and gonococcus, as instanced by their agglutinin and precipitin reactions, as well as their effect on inoculated animals, it seemed interesting to follow the same lines of work and carry them further by testing for possible specificity of the immune bodies developed in the sera of animals immunized to these two organisms. Some of the experiments made by Dr. Flexner[3] on the biology of the diplococcus were repeated with the gonococcus in order to detect possible differences.

BIOLOGY OF THE GONOCOCCUS.

Like the intracellularis, the gonococcus is a coffee-bean shaped diplococcus occurring more frequently within leucocytes than outside them. Comparison of smears from the pus of a recent case of gonorrheal vaginitis and from the cerebro-spinal fluid of an early case of cerebro-spinal meningitis (both in infants) shows a marked similarity, in that both present many polymorphonuclear leucocytes containing from one to ten or more pairs of Gram-negative diplococci, and some pairs of cocci lying extra-cellular. There is, however, a decided difference in the size of the two varieties of organisms, the intracellularis being much the larger of the two. This relatively larger size is also seen in smears made from the peritoneal exudate in guinea-pigs killed by inoculations of diplococcus and gonococcus respectively. In agar cultures, twenty-four hours old, on the other hand, the gonococcus is larger, possibly because its growth is so much less profuse.

[1] Received for publication July 2, 1907.

[2] Brickner and Cristéanu, *Compt. rend. de la Soc. de Biologie*, 1906, lx, 846; 942; 988; 1070.

[3] Flexner, *Jour. of Exper. Med.*, 1907, ix, 105.

Gonococcus grows best upon glucose-serum-agar, prepared by adding about one third its volume of human pleuritic or ascitic fluid to the melted agar. The reaction of the medium proved unimportant unless very alkaline to phenolphthalein, a faintly acid, neutral, or faintly alkaline medium giving about equal growths.

The cultures of gonococcus studied were isolated from cases of vaginitis occurring in young infants at the Babies' Hospital. Smears from the discharge in such cases show the gonococcus to be the only organism present in almost every instance, so that glucose-serum-agar plates made from the pus obtained by passing a platinum loop into the vaginal canal gave, in the majority of instances, an abundant and almost pure growth of gonococcus colonies.

Glucose-agar and dog's serum-agar proved useless as a medium for the cultivation of the first generation of gonococci, but subsequent plants from human serum-agar to these media gave a faint growth consisting of separate colonies surviving from two to five days, and bearing transplantation for from two to six generations only. Sheep's serum-agar proved an excellent culture medium, providing that fully one third volume of serum was added. The sheep's serum-glucose-agar ordinarily used for the growth of *Diplococcus intracellularis* was useless, because it contained too little serum. Thalmann's[4] agar gave excellent growths of all the strains of gonococci, but the cultures survived only from seven to twelve days. Picker[5] found that not all strains of gonococcus grew on Thalmann's agar, but some survived twenty-one days, and some even as long as six months, if the tubes did not become dry. The advantage of Thalmann's agar would seem to lie in its reaction, obtained by neutralizing two thirds of the quantity of agar to phenolphthalein and then adding the other, acid, third. Vannod[6] found that plain agar made slightly alakaline to litmus paper is a very suitable medium for the gonococcus, while if the agar is alkaline to phenolphthalein the organism will not grow upon it. I did not repeat Vannod's experiments.

On human serum-glucose-agar slants gonococci remained viable frow twenty-six to thirty-eight days when capped with rubber and

[4] Thalmann, *Cent. f. Bakt.*, 1900, xxvii, 828; 1902, xxxi, 678.
[5] Picker, *Wien. klin. Woch.*, 1906, xix, 1282.
[6] Vannod, *Cent. f. Bakt.*, 1906, xl, 162; 1907, xliv, 10.

kept at 37° C., or as long as the tubes remained moist. The corresponding uncapped cultures in the thermostat usually survived from sixteen to twenty-one days. The addition of a drop of a suspension of calcium carbonate did not prolong the viability of the gonococcus beyond the period of survival on moist serum-glucose-agar, while the danger of making the medium too alkaline with the carbonate is a drawback to its employment.

Gonococcus did not grow in sheep's serum-water containing sugars, but in human serum-water litmus medium to which dextrose or maltose had been added gonococci caused slight reddening without coagulation. Lactose, saccharose, mannite and dextrine were unaffected. In their ability to ferment sugars, gonococcus and intracellularis acted alike. Dunn and Gordon[7] found that the gonococcus did not affect maltose, thus differentiating the two varieties of cocci. The ten strains I isolated from infants all fermented maltose.

Some of the experiments in viability and autolysis made by Dr. Flexner[8] with diplococcus were repeated with gonococcus, suspensions being made in salt solution and also in Ringer's fluid, a duplicate series kept in the ice chest and in the thermostat at 37° C. The suspensions were of four different strengths: the original turbid suspension, and the same diluted twice, five and ten times. The results were almost parallel with those obtained by Dr. Flexner with the *Diplococcus intracellularis,* more cocci remaining viable in the concentrated salt solution suspensions kept at 7° C., and more in the weaker suspensions at 37° C. While in Ringer's fluid the larger number of cocci survived in the concentrated suspension at 37° C., and in the weaker suspension at 7°C. Cover slips were made daily from these tubes, and the cocci found to be less disintegrated in the lower than in the higher salt solution suspensions kept in the thermostat, while in the tubes kept in the ice chest there was remarkably less disintegration, many cocci staining well on the sixth day, although growth had ceased on the third from both salt and Ringer's solution suspensions. Growth took place after six days in sub-cultures made from the salt solution suspensions kept at 37°

[7] Dunn and Gordon, *Brit. Med. Jour.,* 1905, ii, 421.
[8] Flexner, *loc. cit.*

C., and after seven days from the Ringer's solution suspensions. Cover slips showed rather less daily disintegration in the Ringer's solution than in the salt solution. Among the surviving cocci the larger, more resistant pairs, taking on a deep safranin stain, described by Dr. Flexner, were distinctly seen in the cover slips. As in the case of the intracellularis, cold is more injurious than warmth to the gonococcus. But unlike the intracellularis, Ringer's fluid did not prolong the viability of the gonococcus beyond or even up to the period of survival in ascitic-glucose-agar. Even when kept in the ice chest (at 37° C.) growth was obtained in sub-cultures on the seventh day from the serum-agar tubes, while such tubes kept in the thermostat gave excellent sub-cultures on the twenty-seventh to thirty-ninth days. Cover slips showed many deeply staining cocci at that late day, but most of the cocci had been disintegrated.

Two different strains of the diplococcus grew in sub-cultures made on the thirty-fifth day after inoculation on moist sheep's serum-agar, the tubes having been kept constantly at 37° C. A parallel set of tubes kept in the ice chest gave growth for seven days only and no well-staining cocci could be detected in cover slips made on the eighth day. Thus while gonococcus survives longer on solid media kept at 37° C. than does the *Diplococcus intracellularis,* the viability of both organisms on such media at 7° C. is about the same.

As in the case of the diplococcus, the autolytic ferment of the gonococcus is destroyed by exposure to a temperature of 65° C. for thirty minutes. Suspensions in water, salt solution and Ringer's fluid gave the same results in this respect.

PATHOGENESIS OF THE GONOCOCCUS.

Cultures of gonococcus isolated from cases of vaginitis at the Babies' Hospital were inoculated into white mice and young guinea-pigs. The first or second generation was used to inoculate the surface of a pint Blake bottle of serum-glucose-agar. After twenty-four hours at 37° C. the growth was suspended in four cubic centimeters of 0.9 per cent. salt solution and one half injected into the peritoneal cavity of each of two guinea-pigs weighing between 170 and 200 grams. In this way nine different strains were injected in

the second or third generation. All the pigs died within twenty to twenty-four hours. White mice succumbed to smaller doses of recent cultures, one serum-agar slant being sufficient as a rule to cause death over night, half a tube failing to do this. As the mice reacted very irregularly to the inoculations, they were not used extensively.

Cultures lose their virulence readily. It was found that where a second or third generation had caused death in a guinea-pig (170 to 200 grams) in twenty hours, when given doses of half the surface growth of a pint Blake bottle, the sixteenth or eighteenth generation proved not to produce a fatal result when the entire growth in the bottle was injected.

It has been shown by Bail[9] that sub-lethal doses of bacteria may become lethal under the influence of fluids containing aggressins, so-called, which remove the natural protective powers of the organism. While Bail and Weil[10] maintain that aggressins are formed and found chiefly, though not exclusively, in the body fluids, and first at the point of inoculation where the bacteria are proliferating most rapidly, Wassermann and Citron[11] were able to achieve apparently the same results with bacterial extracts made by shaking cultures in normal rabbit's serum or distilled water; and they insist that aggressins are not newly formed in the animal organism, but are merely a dissolved bacterial substance, which is itself toxic. Bail[12] holds the opinion that the natural aggressins described by him, and the artificial ones obtained by Wassermann and Citron are not identical; but into this discussion I shall not enter.

Only artificial gonococcus and diplococcus aggressins, so-called, were used in my work, the extracts being prepared by suspending the twenty-four hours old growth on the surface of a Blake bottle in five cubic centimeters of salt solution, adding a few drops of toluol and leaving the cocci to autolyze over night at 37° C., after which the resulting fluids were preserved in the refrigerator. Just

[9] Bail, *Arch. f. Hyg.*, 1905, lii, 272.

[10] Weil, *Cent. f. Bakt.*, 1906, xli, 121. Bail and Weil, *Cent. f. Bakt.*, 1906, xlii, 51.

[11] Wassermann and Citron, *Deut. med. Woch.*, 1905, xxxi, 1101. Citron, *Cent. f. Bakt.*, 1906, xli, 230.

[12] Bail and Weil, *Cent. f. Bakt.*, 1906, xlii, 51.

before inoculating, the extract was centrifuged until clear or nearly so, and the toluol removed by evaporation in the thermostat (37° C.). Intracellularis extracts were made with ten cubic centimeters of salt solution because the growths were so much more profuse than those of the gonococcus. Whether the addition of this extract to a sub-lethal dose of the coccus would increase its pathogenicity was tested in two ways: First, non-fatal doses of the coccus and its own extract were used; second, the extract of a recent culture (third generation) was given with the cocci of an old culture (forty-ninth generation) and vice versa.

White mice were used for the cross experiments between old and recent strains. It became apparent that the addition of half a cubic centimeter of extract to a non-fatal dose of a twenty-four hour old culture of its own or the other strain of gonococcus caused the death of white mice within twenty-four hours. Specificity of the aggressin is not limited to the homologous strain of gonococcus.

Working with a strain which did not kill a guinea-pig, weighing 170 to 200 grams, in doses of two cubic centimeters, it was found that the addition of a quarter of a cubic centimeter of its extract made the dose a fatal one. Conversely, two cubic centimeters of the extract proving sub-lethal, the addition of a quarter of a cubic centimeter of a surface growth in a Blake bottle suspended in five cubic centimeters of salt solution caused death within ten to twenty hours. But on decreasing the maximum sub-lethal dose of extract or culture in these combinations the animals did not die regularly within twenty-four hours, so that the invariably fatal dose proved to be two cubic centimeters of suspension of the culture plus one quarter of a cubic centimeter of extract, or vice versa.

Working with a diplococcus which was not fatal in doses of half a cubic centimeter (of a ten cubic centimeter salt solution suspension of a twenty-four hour growth on sheep's serum-agar in a pint Blake bottle), the addition of half a cubic centimeter of the extract of the same coccus caused death within eighteen hours, a smaller dose of either extract or culture proving non-fatal. Nothing less than one cubic centimeter of this extract alone killed over night.

The fatal dose of both the gonococcus and the intracellularis combinations having been determined, cross reactions were made. The

suspensions of intracellularis and gonococcus were always made as nearly equal in strength as possible. It was found that a larger dose of gonococcus culture, two cubic centimeters, was required to make half a cubic centimeter of diplococcus extract fatal, while only half a cubic centimeter of diplococcus culture sufficed to make two cubic centimeters of gonococcus extract kill within twenty hours; more was needed than of intracellularis culture to raise the power of the other organism to the fatal point. Thus it becomes evident that the aggressive action of the extracts of gonococcus and diplococcus is more potent for its own than for the other variety of coccus, though the two may act interchangeably in larger doses, and hence are not specific. Dörr[13] has shown the lack of specificity of many bacterial aggressins (coli, dysentery, cholera, pyocyaneus, staphylococcus), and Paul and Lotti[14] found a certain quantitative but not qualitative specificity among them. Bail[15] and Salus,[16] on the other hand, maintain that the natural aggressins are strictly specific.

To prove whether inoculation with living gonococcus cultures or with their extracts protected against intracellularis cultures and extracts, and vice versa, pigs which survived the above experiments were later given a lethal (or larger) dose of the other organism. The diplococcus extract alone, and also non-fatal combinations of extracts and culture did not protect against a fatal dose of a recent diplococcus culture given five to twenty-eight days later. Sublethal doses of intracellularis culture (0.05 to 0.2 cubic centimeter) protected against a fatal dose given seven to nine days later, while less (0.025 cubic centimeter) did not protect. The gonococcus culture alone, and the culture plus the extract, enabled pigs to survive a fatal dose of diplococci injected thirteen to thirty days later. Combinations of gonococcus cultures and intracellularis extract protected against a lethal intracellularis dose administered two to five days later. Not only is specificity lacking here, but the gonococcus alone or in combination with its extract seems to be a more powerful

[13] Dörr, *Wein. klin. Woch.*, 1906, xix, 759; 1038; 1081.
[14] Paul and Lotti, *Cent. f. Bakt.*, 1907, xliii, 718; 809.
[15] Bail, *loc. cit.*
[16] Salus, *Wien klin. Woch.*, 1906, xix, 870.

protection against fatal doses of living diplococci than the diplococcus itself.[17]

The anatomical lesions found in guinea-pigs dying within twenty-four to thirty-six hours after inoculation with living gonococcus cultures are very similar to those described by Dr. Flexner[18] in pigs which succumbed to intracellularis injections. There are marked œdema of the pancreas and surrounding tissues, congestion or hæmorrhage of the adrenals, small hæmorrhages into the mesentery, serous coat of the intestines and the parietal peritoneum, with more or less clear or turbid fluid in the peritoneal cavity, and a layer of pus and fibrin over the liver, spleen and omentum. An increased amount of clear fluid in the pleural cavities is often noted. Cover slips from the peritoneum and omentum show varying numbers of polymorphonuclear leucocytes and of diplococci, within and outside these cells. Multiplication of the cocci is more in evidence after inoculation with both culture and extract than when culture alone is injected, and phagocytosis is much less marked under those conditions. When the extract alone has been administered neither cocci nor leucocytes appear in the cover slips, and cultures remain sterile.

SERUM REACTIONS.

Precipitins.—Four sera were tested for precipitin reactions. Three were from rabbits immunized to the gonococcus, and one from a rabbit inoculated ten times with the intracellularis. The sera were tested from twenty to twenty-seven hours after bleeding and the cocci were prepared in four different ways: the sodium hydrate (0.15 per cent.) macerations recommended by Brickner and Cristéanu;[19] salt solution macerations prepared in the same way; salt solution toluol extract described by Flexner;[18] and the filtrate of Thalmann's broth used by Torrey.[20] Cultures of diplo-

[17] The reactions just described call for a special study in order to establish their significance. The failure of the *Diplococcus intracellularis* to induce resistance or immunity, may be due to a slower final recovery period than in the case of the gonococcus. The reaction noted of the gonococcus versus the diplococcus may, possibly be of the nature of the non-specific reactions of resistance produced by such an indifferent body as bouillon which also endure for a brief period of time.

[18] Flexner, *loc. cit.*

[19] Brickner and Cristéanu, *loc. cit.*

[20] Torrey, *Jour. of Med. Research,* 1907, xi, 329.

coccus, gonococcus and *Micrococcus catarrhalis* were used, and normal fresh rabbit's serum as control. Only one serum (from a rabbit receiving nine inoculations of living gonococci) gave any precipitin reaction, and that only in dilutions of one to ten and one to twenty for both gonococcus and diplococcus. It gave no reactions with *M. catarrhalis.* The normal serum and salt solution controls were always negative. Two other anti-gonococcus sera and one anti-diplococcus serum gave negative precipitin reactions, yet all these sera showed the presence of immune body as demonstrated by the deviation of complement (*vide infra*). Muir and Martin[21] have shown that the formation of precipitate is not a necessary accompaniment of the phenomena of complement deviation in antisera. Brickner and Cristéanu[19] found the precipitin reactions with gonococcus and intracellularis in anti-gonococcus serum to be identical. Torrey[22] finds an appreciable difference between the two.

Agglutinins were not high in amount in any serum obtained by immunizing rabbits with increasing doses of gonococcus and diplococcus over periods of eight to ten weeks. Six sera were examined: (*a*) agglutinated both gonococcus and intracellularis in dilutions of one to ten before inoculation, and in one to fifty after seven injections of living cocci; (*b*) agglutinated one to twenty before inoculation and both gonococcus and diplococcus in dilutions of one to four hundred after ten injections of living cocci; (*c*) agglutinated one to ten before inoculation; after ten doses of gonococcus extract, gonococci were agglutinated in dilutions of one to one hundred, diplococci only in one to fifty dilutions; (*d*) agglutinated in one to ten before treatment, ten doses of living gonococci and extract injected, after which both gonococcus and diplococcus were agglutinated in dilutions of one to fifty; (*e*) did not agglutinate either coccus before treatment; eleven inoculations of living diplococci developed agglutinins for both gonococcus and diplococcus in dilutions of one to four hundred, one to six hundred was negative; (*f*) agglutinated in dilutions of one to twenty before inoculation, and after nine doses of living gonococci the serum gave positive agglutination with gonococci in dilutions of one to one hundred, and with intracellularis, one to twenty.

[21] Muir and Martin, *Jour. of Hyg.*, 1906, vi, 265.
[22] Torrey, *loc. cit.*

Bruck's[23] statement that inoculations with living cultures produce a serum rich in agglutinins but poor in amboceptors seems to be borne out in only two of the four sera so produced in my experiments. But the sera showing the highest agglutination reactions were both obtained with living cultures. One of them, however, showed the highest amboceptor content of any serum studied. On the other hand, the sera produced by means of inoculations with extracts with or without living cocci showed very low agglutinations.

The anti-diplococcus serum kindly supplied by Dr. Jobling was from a horse which is being inoculated with cultures and extracts of *Diplococcus intracellularis*. It agglutinated with the intracellularis in dilutions of one to one hundred, and gonococcus, one to fifty.

Brickner and Cristéanu[24] obtained exactly the same amount of agglutination with diplococcus and gonococcus with the serum of a horse inoculated with gonococci. Vannod,[25] on the other hand, found a marked difference in the degree to which these two organisms agglutinated in their respective specific sera, and believed that while group agglutinins exist, a large number of specific ones also exist. Torrey's[26] results led him to the opinion that the agglutinins for gonococcus and intracellularis are common in very low dilutions only. As no one of my sera agglutinated its homologous coccus (grown on Thalmann's agar) in dilutions higher than one to four hundred, the contrast with Torrey's positive reactions in dilutions of one to two thousand to one to seven hundred thousand after nine or ten inoculations is very marked.

Deviation of Complement.—Müller and Oppenheim[27] were the first to demonstrate specific anti-bodies in the serum of a male (adult) case of gonorrheal arthritis by means of the complement deviation test, normal human serum being used as controls.

Bruck showed the presence of specific immune bodies for gono-

[23] Bruck, *Deut. med. Woch.*, 1906, xxxii, 1368.
[24] Brickner and Cristéanu, *loc. cit.*
[25] Vannod, *Deut. med. Woch.*, 1906, xxxii, 1984.
[26] Torrey, *loc. cit.*
[27] Müller and Oppenheim, *Wien. klin. Woch.*, 1906, xix, 894.

cocci in the serum of three adult cases of gonorrhœal disease (two females and one male). Agglutinins and precipitins were lacking in all of them. He also called attention to the existence of such amboceptors in the serum of inoculated rabbits.

Vannod[28] used gonococcus nucleo-proteid for the immunization of rabbits, and obtained a serum which agglutinated gonococcus in dilutions of one to three hundred and contained sufficient specific immune bodies to inhibit hæmolysis by deflecting complement when added in proportion of 0.01 cubic centimeter to the serum. Another serum, agglutinating at one to four hundred, prevented hæmolysis when present in a dilution of 0.025 and 0.001 cubic centimeter. He thinks that agglutinins and specific anti-body develop side by side, not independently, as Bruck[29] has stated.

Four sera were tested for specific immune bodies by means of Bordet and Gengou's[30] method of complement deviation in the presence of antigen, using a hæmolytic system as the indicator. The free receptors in the extract (antigen) having been bound to the complement by the amboceptor (specific) present in the immune serum to be tested, the addition (after one hour) of an inactivated lytic serum and its corresponding corpuscles caused no hæmolysis. In the present tests, washed hen's corpuscles in five per cent. solution and rabbit's serum made lytic to hen's corpuscles were used. Fresh guinea-pig serum was employed as complement, in amounts varying from one twentieth to one fortieth of a cubic centimeter. Normal horse serum and normal rabbit serum were used as controls, as three of the immune sera were from rabbits and one from a horse. The immune sera and the extracts were tested with corpuscles alone and with hæmolytic system alone, with and without complement, before being combined with either.

1. Serum 494, from a rabbit inoculated with gonococcus extract in salt solution. This serum inhibited hæmolysis by deviating complement in dilutions of one to fifty, with one tenth of a cubic centimeter of extract (Table I). The results were identical when diplococcus extract was used. Having found the minimum amount of amboceptor necessary to bind the complement to the receptors in

[28] Vannod, *loc. cit.*

[29] Bruck, *loc. cit.*

[30] Bordet and Gengou, *Ann. de l'Inst. Pasteur,* 1901, xv, 289.

the extract and thus prevent hæmolysis, this amount was doubled and the antigen titrated against it. Then it developed that 0.05 cubic centimeter of immune serum deviated complement in dilutions of antigen from 0.1 to 0.002 of a cubic centimeter (Table II); and, going further by titrating the serum against the smaller dose of extract, 0.005 cubic centimeter sufficed to inhibit hæmolysis in the presence of 0.05 to 0.0005 cubic centimeter of immune serum (Table III). Below this dilution hæmolysis was complete. As the results were identical with gonococcus and diplococcus extracts with anti-gonococcus serum, it follows that the amboceptor in that serum was as readily bound to the pre-receptors in the intracellularis extract as to those in the gonococcus extract, and strict specificity is, therefore, lacking. I am indebted to Dr. Jobling for the suggestion to titrate immune serum and antigen successively against double the smallest binding dose of either. In this way smaller amounts of immune body were demonstrable. It was hoped, also, to bring out specific differences between the effect of the two extracts upon the serum, but none such were obtained.

Normal rabbit serum with similar dilutions of antigen showed no inhibition of hæmolysis. The following tables embody the above results.

TABLE I.

Immune Serum No. 494.	Complement Guinea-pig	Antigen		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus.	D. Intracellularis.			
0.1	0.05	0.1	0	0.01	0.05	—
0.05	"	0.1	0	"	"	—
0.02	"	0.1	0	"	"	—
0.01	"	0.1	0	"	"	±
0.005	"	0.1	0	"	"	+
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	±
0.005	"	0	0.1	"	"	+

The sign + means complete hæmolysis; — no hæmolysis; and ± incomplete hæmolysis.

The following controls, nine in number, were made with every series of tests. I give them here instead of repeating them in each

Controls.

No.	Immune Serum.	Guinea-pig Complement.	Antigen.	Anti-hen Serum.	Hen's Corpuscles.	Results.
1	0	0.05	0.1	0.01	0.05	++
2	0.5	0	0.1	0.01	0.05	—
3	0.5	0.05	0	0.01	0.05	++
4	0	0	0	0.01	0.05	—
5	0	0.05	0	0.01	0.05	++
6	0	0	0.1	0	0.05	—
7	0.5	0	0	0	0.05	—
8	0	0.05	0	0	0.05	—
9	0	0	0	0	0.05	—

TABLE II.

Immune Serum No. 494.	Guinea-pig Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus.	D. Intracellularis.			
0.05	0.025	0.1	0	0.01	0.05	—
"	"	0.05	0	"	"	—
"	"	0.02	0	"	"	—
"	"	0.01	0	"	"	—
"	"	0.005	0	"	"	—
"	"	0.002	0	"	"	—
"	"	0.001	0	"	"	+
"	"	0	0.1	"	"	—
"	"	0	0.05	"	"	—
"	"	0	0.02	"	"	—
"	"	0	0.01	"	"	—
"	"	0	0.005	"	"	—
"	"	0	0.002	"	"	—
"	"	0	0.001	"	"	+

TABLE III.

Amboceptor Immune Serum No. 494.	Guinea-Pig Complement.	Antigen.		Anti-hen Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.05	0.025	0.005	0	0.01	0.05	—
0.02	"	"	0	"	"	—
0.01	"	"	0	"	"	—
0.005	"	"	0	"	"	—
0.002	"	"	0	"	"	—
0.001	"	"	0	"	"	—
0.0005	"	"	0	"	"	—
0.0002	"	"	0	"	"	+
0.05	"	"	0.005	"	"	—
0.02	"	"	"	"	"	—
0.01	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	—
0.001	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	+

table. It is hardly necessary to say that only when these controls were correct were the results of the experiments admitted. The quantity of immune serum and antigen varied in each series of controls according to the test to be made. The amount of complement, corpuscles and antigen serum were the same throughout.

2. Serum 495. A second anti-gonococcus rabbit's serum, obtained after ten injections of gonococcus extract and cultures, was found to be anti-hæmolytic without antigen whenever guinea-pig complement was employed. As the serum had been inactivated by heating to 54° C. for thirty minutes, it was thought that anti-hæmolytic substances might have developed under the influence of heat. But on using rabbit complement, hæmolysis was complete, even with 0.2 cubic centimeter of the serum.[31] One tenth cubic centimeter of antigen inhibited hæmolysis in the presence of 0.001 cubic centimeter of immune serum (Table IV). In titrating anti-

TABLE IV.

Amboceptor Immune Serum No. 495.	Rabbit Serum Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.1	0.05	0.1		0.01	0.05	—
0.05	"	0.1		"	"	—
0.02	"	0.1		"	"	—
0.01	"	0.1		"	"	—
0.005	"	0.1		"	"	—
0.002	"	0.1		"	"	—
0.001	"	0.1		"	"	—
0.1	"	0	0.1	"	"	—
0.05	"	0	0.1	"	"	—
0.02	"	0	0.1	"	"	—
0.01	"	0	0.1	"	"	—
0.005	"	0	0.1	"	"	—
0.002	"	0	0.1	"	"	—
0.001	"	0	0.1	"	"	—
1.0005	"	0	0.1	"	"	—

[31] Presumably the guinea-pig serum contained free receptors to which the amboceptor in the immune rabbit serum anchored the complement, thus preventing hæmolysis when the hæmolytic serum was added. On saturating the immune serum with guinea-pig corpuscles over night in the ice chest, and centrifuging the next day, the anti-hæmolytic power of the serum was found to have been lost. The same result was brought about by using rabbit instead of guinea-pig serum as complement.

gen against 0.002 cubic centimeter of immune serum, some differences in the results with the two extracts were noted, the gonococcus being the stronger of the two (Table V).

TABLE V.

Amboceptor Immune Serum No. 495.	Rabbit Serum Complement.	Antigen.		Anti-hen Rabbit's Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intercellularis Extract.			
0.002	0.05	0.1	0	0.05	0.05	—
"	"	0.05	0	"	"	—
"	"	0.02	0	"	"	—
"	"	0.01	0	"	"	—
"	"	0.005	0	"	"	—
"	"	0.002	0	"	"	—
"	"	0.001	0	"	"	—
"	"	0.0005	0	"	"	—
"	"	0.0002	0	"	"	±
"	"	0	0.1	"	"	—
"	"	0	0.05	"	"	—
"	"	0	0.02	"	"	—
"	"	0	0.01	"	"	—
"	"	0	0.005	"	"	—
"	"	0	0.002	"	"	—
"	"	0	0.001	"	"	±
"	"	0	0.0005	"	"	+

3. Serum 4. A third anti-gonococcus serum was obtained from a rabbit immunized with living cocci. Amboceptor for gonococcus was present in a dilution of one to ten thousand in the presence of one tenth cubic centimeter of extract, and one to two thousand for intracellularis. Table VI gives the results of this and further titrations of the extract.

It is evident that the highest amboceptor content for gonococcus was present in the anti-serum obtained by immunizing a rabbit with living gonococcus cultures, agglutinins being also higher than in the other two sera from rabbits immunized with extracts, and with extracts plus cocci. In these high dilutions a distinct difference between the amount of gonococcus and diplococcus amboceptor was apparent, although the two ran parallel until a dilution of one to two thousand was reached.

The anti-diplococcus horse serum obtained from Dr. Jobling inhibited hæmolysis completely in dilutions of one to five hundred with one tenth cubic centimeter of diplococcus antigen, and incompletely with that amount of gonococcus antigen. Doubling the

TABLE VI.

Amboceptor Immune Serum No. 4.	Guinea-Pig Serum Complement.	Antigen.		Anti-hen Serum.	Hen's Corpuscles.	Results.
		Gonococcus Extract.	D. Intracellularis Extract.			
0.1	0.05	0.1	0	0.01	0.05	—
0.05	"	"	"	"	"	—
0.02	"	"	"	"	"	—
0.01	"	"	"	"	"	—
0.005	"	"	"	"	"	—
0.002	"	"	"	"	"	—
0.001	"	"	"	"	"	—
0.0005	"	"	"	"	"	—
0.0002	"	"	"	"	"	—
0.0001	"	"	"	"	"	—
0.00005	"	"	"	"	"	±
0.1	"	0	0.1	"	"	—
0.05	"	"	0.1	"	"	—
0.02	"	"	0.1	"	"	—
0.01	"	"	0.1	"	"	—
0.005	"	"	0.1	"	"	—
0.002	"	0	0.1	"	"	—
0.001	"	"	0.1	"	"	—
0.0005	"	0	0.1	"	"	±
0.0002	"	0	0.1	"	"	+
0.0001	"	"	0.1	"	"	—
0.001	"	0.1	"	"	"	—
0.001	"	0.05	0	"	"	—
0.001	"	0.02	0	"	"	—
0.001	"	0.01	0	"	"	—
0.001	"	0.005	0	"	"	—
0.001	"	0.002	"	"	"	—
0.001	"	0.001	"	"	"	—
0.001	"	0.0005	0	"	"	—
0.001	"	0.0002	"	"	"	—
0.001	"	0.0001	"	"	"	—
0.001	"	"	0.1	"	"	±
0.001	"	"	0.05	"	"	—
0.001	"	"	0.02	"	"	—
0.001	"	"	0.01	"	"	—
0.001	"	"	0.005	"	"	—
0.001	"	0	0.002	"	"	—
0.001	"	"	0.001	"	"	—
0.001	"	"	0.0005	"	"	—
0.001	"	"	0.0002	"	"	±
0.001	"	"	0.0001	"	"	+

inhibitory amount and titrating the two extracts, the results proved to be identical, deviation being complete with 0.02 cubic centimeter of antigen, incomplete with 0.005 cubic centimeter, and absent with a smaller quantity. On testing the inhibitory amount of antigen with diminishing amounts of amboceptor, it was found that 0.01 cubic centimeter was required to prevent hæmolysis with intracellularis, and 0.02 cubic centimeter for gonococcus. Neither the

amboceptor content nor the agglutinins of this serum were high, and the differences for the two varieties of cocci were very small.

Extracts of *Micrococcus catarrhalis,* of *Streptococcus pyogenes,* of a Gram negative diplococcus from a dog, of the typhoid bacillus and of two varieties of dysentery bacilli (Shiga and Flexner types) did not inhibit hæmolysis in combination with this diplococcus immune serum. Normal horse serum controls with gonococcus, diplococcus, streptococcus, *Micrococcus catarrhalis,* and the typhoid and dysentery bacilli were all completely hæmolyzed.

An anti-typhoid serum obtained from Dr. Park at the Board of Health deviated complement in a combination of one tenth cubic centimeter with one two hundredth cubic centimeter of typhoid antigen, but it had no effect upon hæmolysis when combined with intracellularis, gonococcus, streptococcus or *Micrococcus catarrhalis.*

On several occasions extracts of gonococcus and of diplococcus became useless after about two weeks, because they inhibited hæmolysis in doses of one tenth cubic centimeter or less. This was found to be due to the presence of protectin,[32] which fact was demonstrated by shaking the extract with ether for two hours, decanting, evaporating over a water bath and taking up the residue in salt solution. Two tenths and one tenth of a cubic centimeter of this suspension protected blood corpuscles from solution in the presence of their inactivated lytic serum and fresh complement. I am indebted to Dr. Noguchi for this demonstration.

CONCLUSIONS.

The most marked differences, exclusive of pathogenic effects in man, between gonococcus and *Diplococcus intracellularis* are cultural ones, and consist chiefly in abundance of growth and choice of medium.

Relatively larger doses of gonococci than of diplococci are required to kill young guinea-pigs, but the lesions are very similar in the two cases, and both organisms lose pathogenic power rapidly when cultivated artificially.

Agglutinins, aggressins, protective power, and the amboceptors

[32] Noguchi, *Jour. of Exper. Med.,* 1906, viii, 726.

developed in the serum of immunized animals seem to be largely common to both diplococcus and gonococcus.

Neither other Gram negative cocci nor *Streptococcus pyogenes* have any receptors in common with intracellularis and gonococcus.

My thanks are due to Dr. Flexner for suggesting and supervising the work, and to Dr. Jobling for many courtesies.

FURTHER INVESTIGATION UPON THE INFLUENCE OF ORGAN EXTRACTS OF COLD-BLOODED ANIMALS ON THE BLOOD PRESSURE.[1]

By DON R. JOSEPH, M.S., M.D.

(*From the Department of Physiology of St. Louis University.*)

In the spring of 1906, Brown and Joseph,[2] working together, showed that there were either pressor or depressor substances or both present in several organs of various cold-blooded animals. The material collected for that paper was insufficient to make anything more than a very superficial test of the organs used, but during the summer of 1906, Doctor E. P. Lyon, while at Beaufort, North Carolina, and Woods Hole, Mass., kindly collected more material from the same species, and also material not before tested.

The material was preserved in two ways: (1) The fresh organs, such as shark testis, ovary, pancreas and spleen, were cut into thin strips, placed upon filter paper and exposed to the rays of the sun till the water had evaporated and the fat if present in quantity had exuded. The dried material was then wrapped up and shipped immediately to St. Louis, where it was used in a short time. The small amount of oil which remained in the tissue seemed to prevent any deterioration. Before use, this dried tissue was cut into fine pieces and ground up with clean sand in a mortar. (2) Other organs when removed were immediately placed in jars of ninety-five per cent. alcohol, and the jars tightly closed. Before use these organs were put through a meat grinder and ground very fine. They were then spread over a clean glass plate and dried. The alcohol in which the organs had been preserved was evaporated to dryness. The dried residue after this evaporation was added to the dried organ and the whole run through a pulverizer and reduced to a fine powder. No difference in action was observed

[1] Received for publication July 8, 1907.

[2] Brown and Joseph, *Jour. of Physiol.*, 1906, xxxiv.

between the material dried at the seashore and that preserved in alcohol and dried afterwards.

Methods of Preparing Extracts.—The extracts were prepared in various ways. Definite weighed quantities of the dried glands were used in making up the various solutions. Sometimes they were made up to a given percentage in normal saline solution and warmed to 41° C. for fifteen to twenty minutes and then filtered. These were termed "warm extracts." The residue was sometimes made up to its original percentage, either in more normal saline or in distilled water, boiled for from ten minutes to two hours, and then filtered. Such extracts were termed "sediment extracts." Again, a solution was made by boiling a fresh mixture of organ and normal saline for from ten minutes to two hours, then filtering. These were termed "boiled extracts." Extracts were made in ninety-five per cent. alcohol, and also absolute alcohol, filtered, the filtrate evaporated to dryness, and the residue made up to the original percentage in normal saline, so as to make the relative solubilities in physiological salt solution, distilled water, and alcohol apparent by comparing the results obtained upon injecting them into an animal. By the expression "made up to a certain (or original) percentage," I mean this: a three per cent. extract of an organ is one in which three grams of the dried gland were added per hundred cubic centimeters of water. Therefore, a three per cent. extract is the "extract" of three grams of gland in one hundred cubic centimeters of water. In the case of the alcoholic extract, if three grams of gland were added to one hundred cubic centimeters of alcohol, then to the dried filtrate after extraction, would be added one hundred cubic centimeters of physiological salt solution.

Method of Recording Results.—These experiments were all performed upon dogs. The A. C. E. mixture was the anesthetic used in all experiments except when a subcutaneous injection of extracts was made to determine any changes of temperature, in which cases no anesthetic was necessary.

In those cases where tracings of the respiration were taken a "T" tube connected with a tambour was inserted between the tracheal cannula and anesthetic bottle. The blood pressure was recorded by

means of a three-way cannula in the carotid artery, connected with a pressure bottle, and mercury manometer. Sodium citrate (two per cent.) was used to fill the tubing of the blood pressure apparatus. The extracts were injected by means of a burette and a cannula inserted in the central end of the external jugular vein. All injections were made at body temperature. The burette was surrounded by a large glass tube which was kept full of warm water to insure a constant temperature of the injected extracts. In all cases where different extracts were tested upon the same dog, the tubing and burette were disconnected from the venous cannula, and both cannula and burette carefully cleaned out with warm distilled water or physiological salt solution. The moment of injection was recorded by a magnetic signal connected in an electric circuit with the automatic make and break of an induction machine. The time was recorded in seconds by another magnetic signal, and the writing point of this lever was adjusted beforehand so as to show also the line of zero blood pressure. All levers were arranged to write in the same vertical line. Control injections were frequently made by injecting an equal quantity of the physiological salt solution used in making the extracts, but these made it plain that the results obtained when injecting an extract were not due to the salt solution used in making up the extract.

Extracts of the following organs were tested: liver, spleen, pancreas, ovary and testis of shark; liver, kidney, spleen, and testis of swordfish; and spiral valve of dog fish. Care was taken to see that solid particles were absent from the injected substance, except in those cases where the effect of such particles, if present, was under consideration. More than one experiment was performed to determine the different points, but only a single representative effect to show each point will be described. Since the effects of shark and sword fish testes were among the most potent of those examined, an effort was made to determine the following facts regarding them:[3]

I. How small an amount of extract will produce a noticeable physiological effect?

[3] Because of a limited amount of material, however, I have not determined in every case the same point for both shark and sword-fish testis. I might say that in those cases where the same point was determined for each, the results were very similar.

II. Will either the pressor or depressor substance dialyze?

III. Can the pressor and depressor substances be separated:

A. By dialysis?

B. By difference in solubility in absolute alcohol?

C. By difference in solubility in cold normal saline solution?

IV. What is the effect of long-continued boiling?

V. To what physiological change is the marked fall of blood pressure due?

VI. What is the effect of extracts after cutting the vagi?

VII. What is the effect upon respiration?

VIII. What is the effect of subcutaneous injections?

IX. What is the effect of intrastomachic injections?

X. Is the effect of a single extract exactly the same on different animals, or does a personal idiosyncrasy of the animal cause a variation?

Experimental results on these points are as follows:

I. *How small an amount of extract will cause a noticeable physiological effect?*

An extract of shark testis was used in which one cubic centimeter of extract equaled one milligram of the dried gland. An injection of twenty-five cubic centimeters of this extract into a dog weighing eight kilos caused an initial rise of blood-pressure of thirteen millimeters of mercury beginning two seconds after the commencement of injection. In fifteen seconds the pressure had fallen to normal again, and fifteen seconds later was eight millimeters of mercury below normal. The total duration of this depression was thirty seconds. This was the smallest amount of extract used, but it gives a very noticeable though transient effect.

II. *Will either pressor or depressor substance dialyze?*

Method.—An extract of swordfish testis was made as follows: A three per cent. solution of swordfish testis in normal saline was boiled for one hour and forty-five minutes and then filtered through cotton. The filtrate was placed in a dialyzer and dialyzed against a physiological salt solution for five and one half hours.

Results.—Fifty cubic centimeters of the dialysate were injected

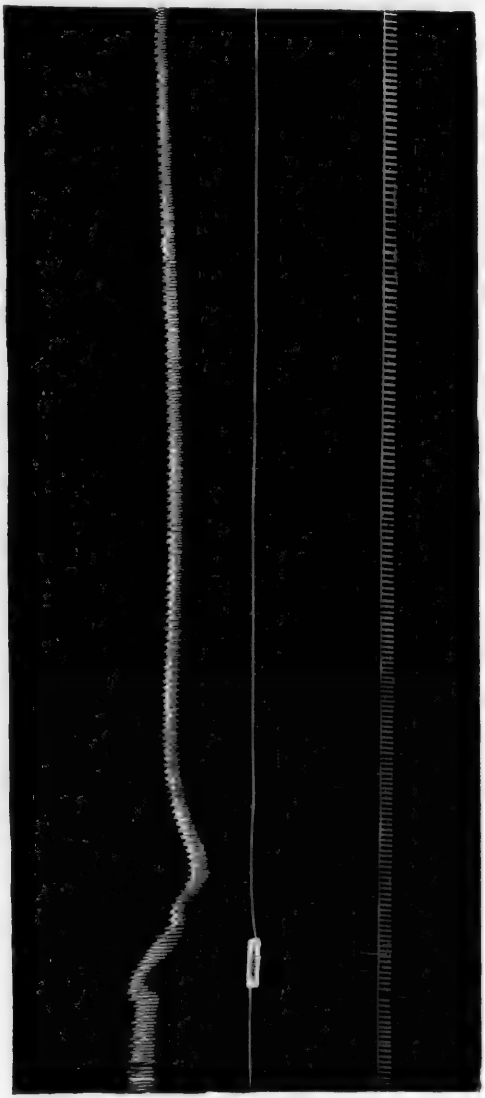


FIG. 1. Injection of 50 c.c. of the dialysate from a three per cent. solution from testis of swordfish, dialyzed against 0.9 per cent. sodium chloride for five and a half hours.

(see Fig. 1) into a dog weighing seven kilos. At the beginning of the injection the blood-pressure was 102 mm. of mercury. In twenty-one seconds the pressure had fallen 23 mm. The return of pressure to normal was, for a few seconds, rather rapid, but became slower as it rose, and the normal level was not regained for two and two thirds minutes. There was practically no pressor effect shown. A control injection of an equal quantity of the physiological salt solution used in making the extracts gave neither pressor nor depressor effects. It is, therefore, concluded that the depressor substance will dialyze, but that the pressor very probably will not.

III. *Can the pressor and depressor substances be separated?*

A. *By Dialysis.*

Method.—A three per cent. solution of swordfish testis in 0.9 per cent. sodium chloride solution was warmed at 41° C. for thirty-five minutes, and after standing for some time until solid particles had settled, it was filtered through cotton. The filtrate, which at that time showed the presence of both pressor and depressor substances, was divided into two portions (*a*) and (*b*); (*a*) was put upon ice as it was; (*b*) was put in a parchment tube for dialysis, surrounded by normal saline, and placed upon ice also. Once every twenty-four hours both (*a*) and (*b*) were heated to 76° C. in a water bath, and (*b*) placed in a fresh parchment tube with fresh normal saline surrounding it. This process was kept up for five days, when the extracts were tested.

Results.—(*a*) An injection of twenty-five cubic centimeters of this solution (see Fig. 2) lowered the blood-pressure from 114 mm. to 20 mm. Hg in forty-eight seconds, and it was impossible to get a recovery of the animal. An injection of five cubic centimeters of the same extract into another dog caused the following results: The blood-pressure fell 23 mm. Hg in seventeen seconds, and seven minutes afterward it was not quite back to the level existing at the time of injection. The rate of heart beat just before injection was 115 per minute. One minute afterwards 96 per minute, and four minutes after injection the normal rate of beat had been reëstablished. There was no evidence of the presence of a pressor substance.

(*b*) An injection of fifteen cubic centimeters of this solution into the same dog used for the latter part of (*a*) above caused a fall of 22 mm. Hg, which lasted about four minutes. There was almost no evidence of the presence of a pressor substance. The heart

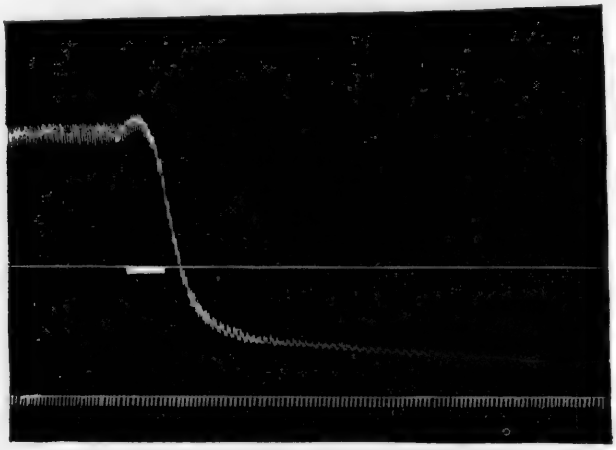


FIG. 2. Injection of 50 c.c. of a three per cent. extract of testis of swordfish in 0.9 per cent. sodium chloride prepared at 41° C. for thirty minutes.

rate was 158 just before injection, 183 at the point of lowest blood-pressure, and 152 three minutes after injection.

Apparently it is not practical to try to separate the two active substances in this way, inasmuch as the pressor substance is lost or destroyed by the method used. It may have been destroyed by the repeated heating to 76° C., the long standing in watery solution, or (possibly though not probably) by bacterial action. I do not think the cause was bacterial action, since no putrefactive processes were detectable. The depressor action of equal doses of (*a*) and (*b*) is considerably less prominent in the case of (*b*). This is probably the result of removal of a considerable amount of depressor substance by the long-continued dialysis of solution (*b*).

B. *By difference in solubility in absolute alcohol.*

Method.—It has been found in organ extracts of warm-blooded animals,[5] that the depressor substance is soluble in absolute alcohol and the pressor substance insoluble. To see if this would hold

good for these cold-blooded animal organ extracts, solutions were made as follows: A three per cent. solution of sword fish testis in absolute alcohol was made and left standing at room temperature (21° C.) for fifty minutes. It was then filtered through cotton, the filtrate evaporated to dryness and the residue, after evaporation, made up to three per cent. in normal saline. This will be called solution (*a*), and if the results are the same as in the case of warm-blooded animals it should contain depressor and no pressor substance (or at least a less amount of pressor than the normal). The original residue remaining after filtering through cotton was dried, made up to three per cent. in normal saline, boiled for one hour, and then filtered through cotton. This will be called solution (*b*), and it should give pressor and no (or little) depressor effect.

Results.—Without giving exact figures for the effects of these two solutions, I will say that it does not seem possible to separate the two active substances in this way. The pressor effect is about equal in each solution. The depressor action is, however, only half as strong in the absolute alcohol extraction as in the physiological salt solution, seeming to indicate that the depressor at least is less soluble in absolute alcohol than in physiological salt solution.

C. *By difference in solubility in cold normal saline.*

Method.—A four per cent. solution of sword fish testis in 0.9 per cent. salt solution was allowed to stand for seven minutes at room temperature (21° C.) and then filtered. The filtrate was called solution No. 1. The residue was again made up to four per cent. in more normal saline and allowed to stand at room temperature for twenty-five minutes, to see if most of the pressor substance had been dissolved out in solution No. 1. The filtrate this time was called No. 2. The residue made up to the original percentage again, was boiled for some time and then filtered. The filtrate was called solution No. 3.

Results.—The results from this method also are negative. Both substances are found in all the solutions. The depressor substance, however, seems to overshadow the pressor in every case. I believe that the depressor substance is not very highly soluble in water, for I have noticed repeatedly that injections of solutions made up

as (1), (2) and (3) above show almost the same amount of depressor action, and also, that if the length of time that the normal saline is allowed to stand upon the powdered organ is varied, there is not as a rule, a corresponding difference in the amount of depressor action. This is true particularly if the saline is exposed to the organ for longer than about fifteen minutes.

IV. *What is the effect of long continued boiling upon the pressor and depressor substances?*

Method.—A three per cent. solution of swordfish testis was made up in 0.9 per cent. salt solution by warming to 41° C. for thirty-five minutes and filtering. This filtrate was divided into two portions, (1) and (2). No. 1 was left standing unchanged, while No. 2 was boiled for two hours.

Results.—These two solutions, when injected, gave approximately the same amount of fall of blood-pressure, but there was a very marked difference in the length of time the effect lasted in the two cases. Three minutes after injecting No. 1 (see Fig. 3) the pressure had regained a normal level. At the time of injecting No. 2 (see Fig. 4) the pressure was 108 mm. Hg. Immediately following the injection there was the usual sudden fall of blood pressure, but the recovery, though gradual, was exceedingly slow, so slow in fact that twelve minutes after the injection the pressure was only 98 mm. Hg. It was still gradually rising and apparently would have reached the normal level in three minutes more. In No. 2 there was no apparent pressor action, while in No. 1 there was an initial rise of pressure equal to 10 mm. Hg. In neither (1) nor (2) was there any marked effect upon the respiration. The heart rate in (1) was 178 just before injection, 170 at point of lowest pressure, and 174 about two minutes after injection. In No. 2 the rate at time of injection was 123, at point of lowest pressure 101, and twelve minutes after injection 121 beats per minute.

The most apparent thing shown by this line of experiments, is that the pressor substance is rendered inactive by long continued high temperature. The more prominent depressor action following continued boiling of an extract may be interpreted either as a result of the disappearance of the pressor substance alone, or to

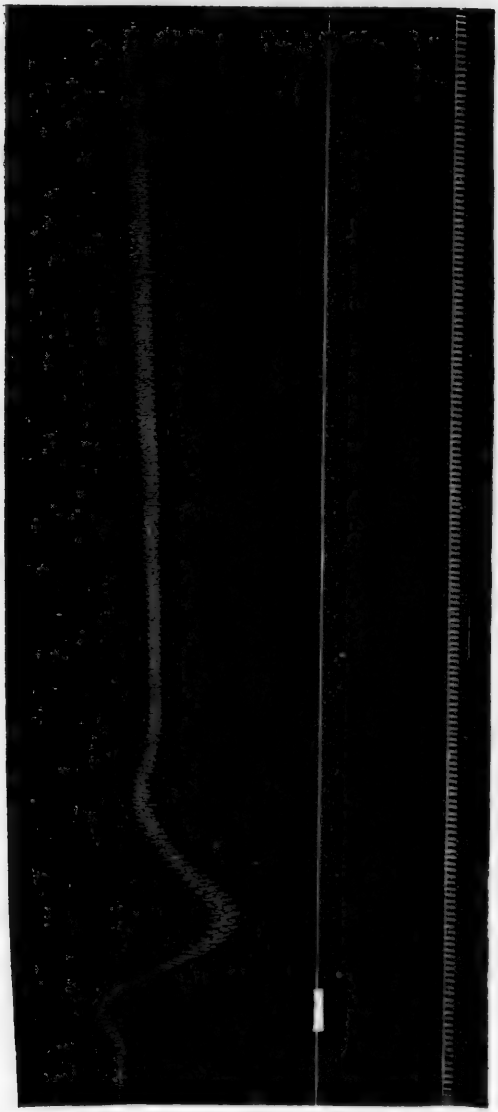


FIG. 3. Injection of 50 c.c. of a three per cent. extract of testis of swordfish, showing effect when extract was boiled for twenty minutes.

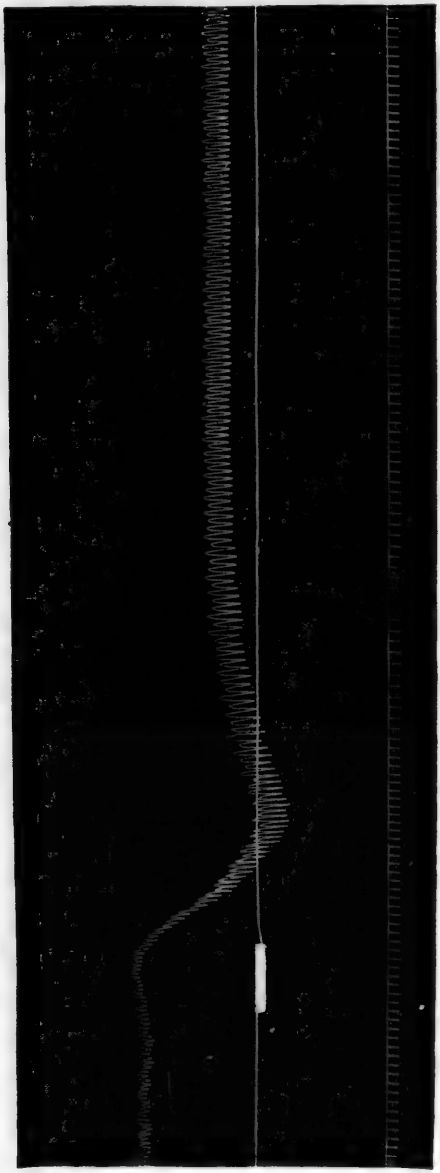


FIG. 4. Injection of a three per cent. extract of testis of swordfish, showing the effect when the extract was boiled for one hour and three quarters. In twelve minutes the recovery was not complete.

that disappearance coupled with the development of another depressor substance not before present.

V. *To what physiological change is the marked fall of blood pressure due?*

Method.—A hind foot and part of the leg of a dog were enclosed in a Mosso's plethysmograph during the time that intravenous injections of extracts were being made.

Results.—An increase in the volume of the enclosed limb followed an injection of twenty-five cubic centimeters of a boiled extract of shark testis. Since in this extract the depressor action is so much more prominent than the pressor, I think it is safe to say that the result obtained, *i. e.,* the vaso dilation was due to the action of that substance. No experiments were performed to determine whether these effects were due to action on the nerve centers, or on the blood-vessels direct.

VI. *What is the effect of extracts after cutting vagi?*

Results.—After cutting both vagi (see Fig. 5) the chief effect noticed is that there is a much more rapid return of pressure to

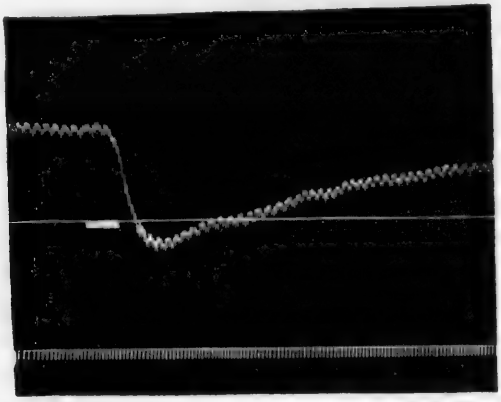


FIG. 5. Vagi cut; injection of 50 c.c. of a three per cent. extract of testis of swordfish (boiled extract). This injection followed the one shown in Fig. 4 and shows the difference in the curve obtained after cutting the vagi.

normal than when the vagi were intact. The fall is about as great as before, but where normally it took three minutes to recover, it

now takes but little over one minute. The rate of heart beat was little affected either before or after cutting the vagi. I am therefore unable to account for the quick recovery after injecting with vagi cut.

VII. *What is the effect upon respiration?*

Results.—I may say at the beginning that I have been unable to reproduce with these extracts the remarkable results which Doctor Brown and I obtained last year from "Sex-gland of Shark." In the case of which I speak an injection of twenty-five cubic centimeters of extract of sex-gland of shark increased the rate of respiration from forty-eight per minute before the injection to eighty-four one minute after injection. There was also a remarkable increase in the heart rate (from 132 per minute to 264 per minute). I cannot explain last year's results in the light of the effects I have obtained since that time. It was unfortunate that we had so little tissue on hand at the time that work was done. We have been unable to learn either the species or sex of the shark whose sex-gland was examined last year. I have since frequently noticed a slightly increased respiratory rate at the time, after an injection when the pressure was lowest, but I have considered it as a natural result of the low blood-pressure.

VIII and IX. *What are the effects of subcutaneous and intra-stomachic injections?*

Results.—I have been unable to get any noticeable effect upon the blood-pressure, heart-rate, or respiration, as a result of injecting by these means. Apparently the absorption is too slow to give any result, or else the substances are changed before reaching the blood-stream.

X. *Is the effect of a single extract the same on different animals, or does a personal idiosyncrasy of each animal cause a variation?*

Method.—A considerable amount of a three per cent. solution of swordfish testis was made up in 0.9 per cent. salt solution and tested upon three dogs.

Results.—In every case the general effect was similar. Individual parts varied slightly. The variation was more marked in the "first" injection in each dog than in the succeeding injections. I also found that where a number of injections were made into

the same dog, the effect of a given dose became less and less pronounced with each injection after the "first" injection. In a few dogs there was a very slight and transient response from the blood-pressure, following the ninth or tenth injection of extract. I had thought that possibly some individual variation due to the animal itself might account for the results which we obtained last year.

The remaining organs were studied only in a preliminary way, and a brief description will be given merely to show their general action.

I. *Shark Organ Extracts:*

(*a*) *Shark Ovary.*—The extracts were made from ovaries containing well developed eggs, and were used in solutions as strong as five per cent. They were tried repeatedly, but no effect worth recording was obtained. This result would indicate that the "Sex-glands of Shark" which Doctor Brown and I used last year was the male organ and not that of the female.

Our records at that time did not show which we were using.

(*b*) *Shark Spleen.*—An injection of twenty-five cubic centimeters of a two and one half per cent. solution of shark spleen in 0.9 per cent. salt solution shows no initial pressor effect. The fall of blood pressure is very rapid, and in this particular case amounted to 54 mm. Hg. The recovery is also very rapid—the normal level being regained in fifty-five seconds after injection. The respiration was not noticeably affected either in rate or depth. The heart rate at commencement of injection was 87 per minute, at the point of lowest pressure 110 per minute, and one minute after injection had slowed down to 104 per minute.

(*c*) *Shark Liver.*—There is nothing particularly interesting to say about extracts of this organ, except that there is a depressor substance present, as in the other organs already described. The effect produced by extracts of this organ is even more transient than in the case of shark spleen. Twenty-five cubic centimeters of a five per cent. solution caused a 42 mm. drop of blood pressure, and in twenty-eight seconds the pressure had returned to its normal level. The respiratory rate was practically unchanged throughout. The heart rate was 132 just before injection, 138 at point of lowest pressure and 156 one minute after injection.

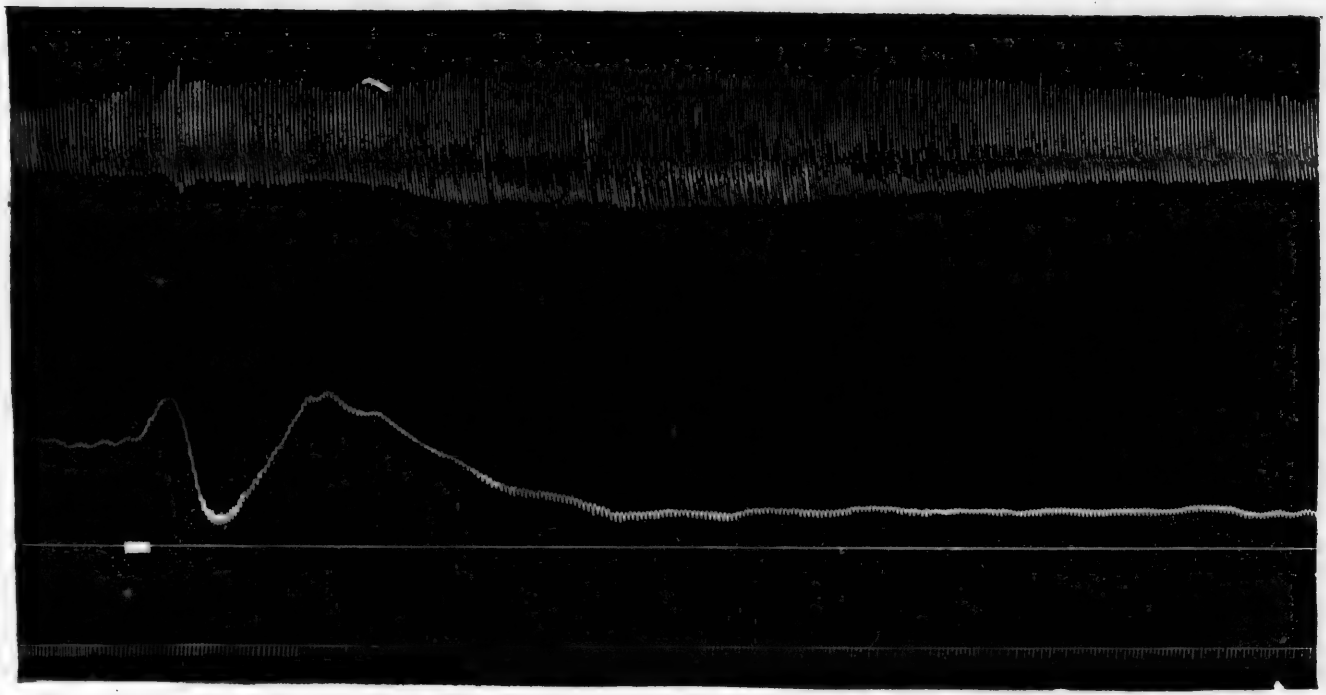


FIG. 6. Injection of 35 c.c. of a two and one half per cent. warm extract of pancreas of shark.

The heart in this case was slower than usual in resuming its original rate.

(*d*) *Shark Pancreas.*—This is one of the most interesting of the organs tested. An injection of twenty-five cubic centimeters of a 2.5 per cent. solution in normal saline (see Fig. 6) caused first an initial rise of blood pressure of 18 mm. Hg, ten seconds elapsing before normal level was regained; second, a fall of 40 mm. which was not recovered from for twenty seconds. In recovering, the pressure did not stop at the normal level, but continued to rise until it was at a height equal to that caused by the initial pressor effect. From this point the pressure gradually fell until in sixty-five seconds it was at the lowest level attained by the first depression. Here the pressure remained (40 mm. below normal) without beginning to rise for over four minutes. The heart rate before injection was 72, during the first depression 90, and during the second depression, two minutes after injection, 66 beats per minute. The rate of respiration before injection was 54, immediately succeeding the injection 66, and two minutes after injection (when the heart rate was 66) 120 per minute. The depth of respiration was almost doubled at the time when its rate was most rapid.

These results might be explained by supposing that there are present two pressor substances and either one or two depressors. If we suppose two pressors and one depressor the order of action would be as follows: The first pressor causes the initial rise, and the depressor action following close upon that of the pressor overcomes the pressor and causes the initial fall. Next the second pressor action shows up and for a few seconds it overcomes the depressor action and causes the second rise in blood pressure. The effect of this second pressor substance is quite transient, and as it wears off the depressor substance resumes its action and holds the pressure at a low level for some time.

II. *Swordfish Organ Extracts:*

(A) *Swordfish Liver.*—Not essentially different from shark liver.

(B) *Swordfish Spleen.*—Not different from shark spleen except that the depressor substance appears to be a little less potent.

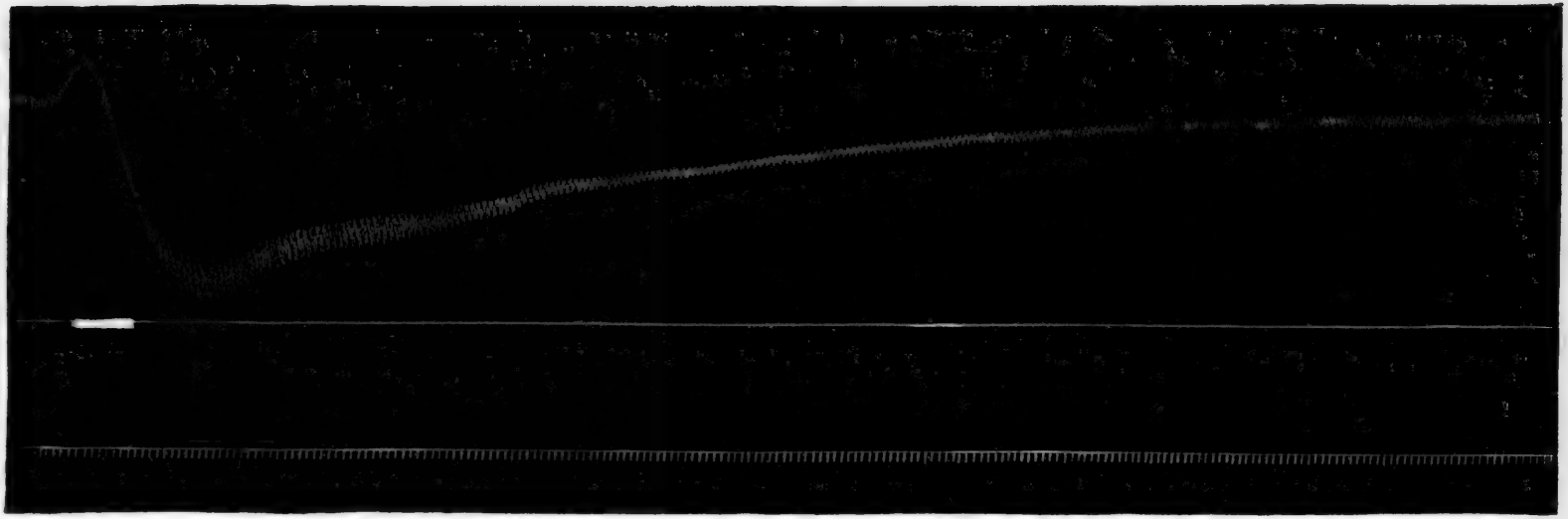


FIG. 7. Injection of 50 c.c. of a three per cent. boiled extract of spiral valve of dogfish.

(C) *Swordfish Kidney.*—This organ also contains a depressor substance. It is not, however, potent enough to warrant a definite description of its effects, except, perhaps, to say that recovery from twenty-five cubic centimeters of a three per cent. solution of boiled extract takes over three and one half minutes. The fall of blood pressure is not very great.

III. *Spiral Valve of Dogfish.*

This organ furnishes an interesting extract. It contains both pressor and depressor substances. An injection of fifty cubic centimeters (see Fig. 7) of a three per cent. solution of spiral valve in 0.9 per cent. salt solution (boiled one and a half hours) produced an initial rise in blood pressure of 18 mm. Hg, followed by a fall of 80 mm. Hg. The recovery was very gradual and it took over four minutes to accomplish it. The only difference between the effect of this boiled extract and a "cold" extract, made by allowing the powdered organ and physiological salt solution to stand at room temperature for twelve minutes, is that the depressor action is more permanent in the case of the boiled extract. Recovery occurs in the case of the cold extract in one and a half minutes. The heart rate was 180 just before injection, 210 when the pressure was lowest, and had practically returned to 180 by the time the normal blood pressure was regained.

SUMMARY.

1. None of the extracts examined are very potent in small doses. A noticeable effect, however, is produced upon the blood pressure by the extract of fifteen milligrams of shark testis.

2. A small dose of a five per cent. shark testis extract seems to show more pressor action than a larger dose of the same solution.

3. The fall of blood-pressure is due to a vaso-dilation.

4. After repeated (seven or eight) injections of almost any extract, an injection of more of the same extract, or of another extract, which would be rather potent as a "first" injection, causes very little if any response from the blood-pressure.

5. Continued boiling of an extract destroys the pressor substance.

6. The depressor action is then very pronounced and especially prolonged.

7. The depressor substance will dialyze.

8. The pressor and depressor substances cannot be separated by dialysis, because in the repeated sterilizations the pressor substance disappears.

9. The pressor and depressor substances cannot be separated by absolute alcohol, as each seems to be soluble. The solubility is not, however, very great, at least in the cold.

10. It seems impossible to separate the pressor and depressor substances by means of a difference in solubility in cold physiological salt solution, since the depressor substance is even more soluble in that solution than the pressor. And yet the depressor substance is not nearly all removed by this means, even though it is allowed to stand for some time.

11. After cutting the vagi, the recovery from the effect of the depressor substance is much more rapid than with the vagi intact. The amount of fall of blood-pressure is about equal in either case.

12. Extracts of the testis and pancreas of shark; of the testis and spleen of swordfish; and the spiral valve of dogfish show the presence of a pressor substance.

13. Extracts of the testis, spleen, pancreas and liver of shark; of the testis, liver, spleen and kidney of swordfish, and the spiral valve of dogfish show the presence of a depressor substance.

14. Extracts of shark ovary gave no result. I believe, however, that if sufficient of the parenchymatous tissue of the ovary had been obtained a result would have been given by this organ also.

A Comparison of the Properties of Organ Extracts of Cold- and Warm-blooded Animals.

Cold-blooded Animals.	*Warm-blooded Animals.*
1. Most organ extracts show the presence of both pressor and depressor substances.	Same.
2. The pressor substance is best obtained by extracting with normal saline at the room temperature, but this is not a very successful means of separating the pressor and depressor substances.	Vincent and Sheen,[4] state that this is the best way to *obtain* and *separate* the two.
3. The pressor action is more transient than that of the depressor.	Same.[4]
4. Boiling, if continued for any length of time, destroys the pressor substance.	Same.[4]
5. Long continued boiling causes a more prominent depressor action either by forming new depressor substances or by removing the pressor.	Same.[4]
6. The depressor substance is much more prominent in its action than the pressor and is probably present in larger amounts.	Same.[4]
7. The fall in blood-pressure seems to be due to a dilatation of peripheral vessels.	The fall of blood-pressure is due to a direct action upon the blood-vessels and not to an action upon the vaso-motor center.
8. The effects of "second" and later injections are not the same as those produced by "first" injections.	Same.[5]
9. Both pressor and depressor substances are soluble in absolute alcohol, and they cannot be successfully separated in this way.	According to Schäfer and Vincent,[5] the pressor substance is insoluble in absolute alcohol and the depressor soluble. They used this means to separate the two.
10. The depressor substance is dialyzable.	Same.[5]
11. No effect is obtained by injecting subcutaneously.	Same.[6]
12. The only difference in the effect of injections made before and after cutting the vagi, is that the normal blood pressure is regained sooner after cutting the vagi than it was before cutting.	No difference in the action of extracts injected before and after cutting vagi.

[4] Vincent and Sheen, *Jour. of Physiol.*, 1903, xxiv, 242.
[5] Schäfer and Vincent, *Jour. of Physiol.*, 1899, xxv, 87.
[6] Osborne and Vincent, *Jour. of Physiol.*, 1899, xxv, 283.